PUBLIC HEALTH ASPECTS OF THE HOUSE FLY, *Musca domestica* L. (DIPTERA: MUSCIDAE) - *Enterococcus* spp. ASSOCIATION

by

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M. Phil., UNIVERSITY OF DHAKA, BANGLADESH, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

DEPARTMENT OF ENTOMOLOGY
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Abstract

House fly (*Musca domestica* L.) larvae develop in decaying organic substrates such as animal manure and adult flies likely play an important role in the ecology of fecal bacteria, including potentially virulent strains. House fly larval development strictly depends on an active bacterial community in the habitat. Although the principle of this symbiosis is not well understood, this association plays a fundamental role in transmission of microbes by this insect. In this study, enterococci were chosen as a model organism to assess the role of house flies in dissemination of multi-drug resistant bacteria in the agricultural environment. House flies (FF) and cattle manure (FM) from a cattle feedlot (frequent use of antibiotics) and house flies (BF) and manure of the American bison (BM) from the Konza Prairie Nature Preserve (no antibiotic use) were collected and analyzed. Results showed a significantly higher prevalence of enterococci resistant to tetracycline and erythromycin in FM and FF compared to that of BF and BM. Enterococcal diversity did not indicate the house fly development in manure in the corresponding habitats but the antibiotic resistance data showed very similar profiles among isolates from flies and corresponding locations. Resistance genes (*tetM, tetS, tetO, ermB*) and the conjugative transposon Tn*916* were the most commonly detected determinants from resistant isolates from both environments. The house fly digestive tract was evaluated for the potential for horizontal transfer of antibiotic resistance genes among *Enterococcus faecali*s. Horizontal transfer of the pCF10 plasmid with the tetracycline resistance gene (*tetM*) occurred in the fly digestive tract with a transfer rate up to 10^{1} T/D. In addition, eight enterococcal species were selected to evaluate their role and survival during house fly development. Overall, the survival rate (egg to adult) was significantly higher with *E. hirae*, *E. durans* and *E. avium* compared to other strains. These results indicate: a) house flies play an important role in the ecology of

antibiotic resistant enterococci; b) the house fly digestive tract provides conditions for horizontal gene transfer among enterococci, and c) enterococci support the house fly development and can colonize the gut of newly emerging adult flies.

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Dedication

To my father, Dr. Md. Khurshidul Islam Chowdhury, my mother Reaza Akter and my father-in-law Dr. Matiur Rahman Chowdhury.

CHAPTER 1

Literature Review

1. The house fly (*Musca domestica* L.)

1.1. Biology, habitat, and behavior

The house fly (*Musca domestica* L.) belongs to the order Diptera, family Muscidae (Moon, 2002; Triplehorn and Johnson, 2005). They are 6-9 mm long, gray and black colored flies. From the public health point of view, house flies are probably the most important nuisance insect pest and mechanical vector of pathogens (Graczyk et al., 2001a). House flies occur on all continents except Antarctica and have a complete (holometabolus) metamorphosis. Their life stages include egg, larva, pupa and adult. Females flies lay eggs directly in the larval developmental habitat and the larval stages consist of three instars. Larvae are mobile and capable of crawling and burrowing into various decaying organic substrates in search for food. Larvae possess porous pharyngeal ridges in their cephalopharyngeal skeleton that is used to filter essential food particles and bacteria from the liquid substrates (Dowding, 1967; Moon, 2002). The 3rd larval instar stops feeding upon reaching maturity and voids all food contents from the digestive tract before pupation. The pupa stage is non-feeding and immobile and encased in a puparium. During the pupation process, the 3rd larval integument becomes hard and forms the puparium (Frankel and Bhaskaran, 1973; Moon, 2002). Adult house flies are non-biting, they have a sponging type of mouthparts. The labellum of mouthparts consists of prestomal teeth on the surface of its lobe which is used for food scraping. Labella also contain chemical and mechanical receptors (Elzinga and Broce, 1986; Moon, 2002). The house fly digestive system consists of the salivary glands, foregut (esophagus, diverticulum, proventriculus), midgut, and hindgut. Adults feed on liquids high in carbohydrates. Though house flies are attracted to a variety of food materials, labella of sponging mouthparts allow them to feed only on liquid food whereas solid foods are liquefied by means of regurgitated saliva (Moon, 2002). For digestion,

food moves through the foregut then midgut. Food digestion mainly takes place in the midgut. After intake, some foods are temporarily stored in the diverticulum, which can be regurgitated and retaken. All undigested food particles and wastes move through the hindgut and is excreted by the anus (Moon, 2002).

House flies require an active microbial community for larval development (Spiller, 1964, Schmidtmann and Martin, 1992; Zurek et al., 2000). Consequently, larvae can be found in a wide variety of decaying organic substrates rich in microbes. House flies oviposit on and develop in decaying organic matters such as animal manure, human garbage dumps, open privies, animal bedding, soil bedding, poultry litter, and also in wastes around food and vegetable processing plants that have a diverse and active microbial community (Greenberg, 1973; Graczyk et al., 2001a; Moon, 2002). Clearly, livestock confinement facilities are the primary house fly developmental habitat (Skoda and Thomas, 1999). Urban refuse such as wastes from meat processing plants can also be the breeding sites for house flies. Several habitats have been investigated in more details to characterize the developmental sites of house fly larvae (Campbell and McNeal, 1979; Meyer and Petersen, 1983). These include cattle manure, manure mixed with soil or hay, and feed of cattle. House fly larval development can occur in fresh manure, approximately 2-5 days old. The average colonization period for fly larvae in cattle manure is 22 days and fly population size fluctuates with the season (Broce and Hass, 1999). House flies exhibit broader preferences for age of the manure, moisture, and temperature range for development than that of other muscoid flies (Stafford and Bay, 1987; Fatchurochim et al., 1989).

House flies can develop throughout the year in tropical countries but not in winter in temperate regions. In North America, they develop throughout the year in the southern parts

where the temperatures remain above freezing. The house fly developmental time (from egg to adult) takes only 14 days under optimum conditions in summer (Moon, 2002). The optimum temperature to complete the life cycle is 27-32^{o}C and no development occurs below 10^{o}C. House flies are multivoltine and one of the most fecund filth flies. Average longevity for female flies is 15-25 days and they lay 5-6 batches of eggs (120-150 eggs/cycle) in their lifetime. Under temperate climates, the number of generations ranges from 10 to 12 in summer (Skidmore, 1985; Hedges, 1990; Moon, 2002). House flies resume the reproductive cycle inside cattle barns, poultry houses and other indoor animal facilities in winter time.

Adult house flies aggregate around garbage, compost piles and enter buildings. They can easily get access inside and start crawling on human food or resting on walls, windows and ceilings (Moon, 2002). House fly adults are mostly active during the daytime when they are engaged mostly in flying, finding the food, feeding, mating, and ovipositing. House flies show random dispersal pattern. They exhibit less movement in areas or farms that have more breeding sites (Broce, 1993). House flies generally do not fly more than 4 km but they are capable to disperse as long as 35 km (Murvosh et al., 1966; Hedges, 1990; Iwasa et al., 1999)

1.2. Digestion of bacteria in the house fly digestive tract

The contribution of microbial community for the larval development of muscoid flies including house flies (Schmidtmann and Martin 1992; Zurek et al., 2000), stable flies (Lysyk et al., 1999, Romero et al., 2006), horn flies (Perotti et al., 2001), and face flies (Hollis et al., 1985) has been investigated. These studies have clearly demonstrated the dependence of muscoid fly larvae on active bacterial communities using artificial or natural media. House fly larval development occurs on artificial substrates such as egg yolk agar (Watson et al., 1993; Zurek et

al., 2000) and blood agar (Schmidtmann and Martin, 1992) inoculated with selected bacteria as well as natural substrates such as cattle manure (Hogsette, 1996) and poultry manure (Moon et al., 2001) that were sterilized by autoclaving and inoculated by bacterial isolates. Clearly, no muscoid fly larval development occurs in sterile substrates (Gerberich 1948; Schmidtmann and Martin 1992; Zurek et al., 2000). The principle of this dependence is not known; however, these data indicate that bacteria support larval development by providing some essential nutrients (e.g vitamins) (Silverman and Silverman, 1953; Schmidtmann and Martin, 1992; Zurek et al., 2000). Several reports have suggested that non-pathogenic bacteria might serve as a primary nutritional source for house flies (Levinson, 1960; Silverman and Silverman, 1953; Watson et al., 1993) whereas pure culture of pathogenic bacterial strains (eg. *Serratia marcescens*) are detrimental to larval development (Zurek et al., 2000). The midgut of fly larvae is capable to lyse the ingested bacteria with several enzymes such as cathepsin-D protease, lysozyme, and acidic pH (Lemos and Terra, 1991; Zurek et al. 2000). Salivary enzymes of house fly larvae such as amylase, maltase and trehalase enzymes show hydrolytic activities. The pH varies in three different regions of the larval midgut (Espinoza-Fuentes and Terra, 1987). The pH is 6.1 in the fore-midgut, 3.1 in the mid-midgut and 6.8 in the hind-midgut. Digestion mainly takes place in the mid-midgut region with acidic pH. Midgut enzymes contain hydrolase, lysozyme, pepsin, alkaline phosphatase, aminopeptidase, γ-glutameal transferase and trehalase (Espinoza-Fuentes and Terra, 1987). The larval digestive system exhibits several physiological adaptations to kill and digest bacteria. First, carbohydrates are degraded in the fore-midgut by amylase activity which is secreted from salivary glands, cells of midgut caeca and fore-midgut. Bacteria in the gut become more vulnerable to the actions of pepsin, lysozyme and acidic pH when the carbohydrate concentration is low (Espinoza-Fuentes and Terra, 1987). After being digested or killed in the

mid-midgut, remaining bacteria reach the hind-midgut. Proteins/ bacteria are mainly digested in the hind-midgut by the action of trypsin (Espinoza-Fuentes and Terra, 1987). In the hind-midgut, all the digestion takes place inside of the peritrophic membrane. Moreover, the secretions of larval salivary glands and hindgut, amylase and maltase can act outside the host body. Their dual action includes liquefication of solid food and digestion of starch in surroundings which make the glucose available for larvae as a nutritional supplement (Espinoza-Fuentes and Terra, 1987). However, the nutritional value of different bacterial species for different species of muscoid larvae is variable (Lysyk et al., 1999; Zurek et al., 2000; Romero et al., 2006).

1.3. Pathogen transmission mechanisms of the house fly

House flies can carry bacteria in their digestive tract or on the body surface (Moon, 2002). House flies are highly mobile and they can spread bacteria by mechanical contact with the substrates. Because of electrostatic charges, the setae and hairs on the body surface of flies have higher capacity to attach foreign particles. The viscosity of feces enhances the adhering capacity of pathogens/foreign particles to the fly body (Graczyk et al., 1999a). Moreover, the pulvillus structure on the fly legs contains sticky substances that accelerate the chances to adhere viruses, bacteria or protozoan cysts (Hedges, 1990). House flies can potentially contaminate any substrate by the unique regurgitation type of feeding and fecal excretion. The pathogen transmission mechanism involves feeding on fecal material with pathogens, ingestion and regurgitation of pathogen contaminated digestive fluids or fecal excreta on human food which is eventually ingested by people (Osato et al., 1998; Graczyk et al., 2001a). Ingested viruses or bacteria can retain their virulence in the fly gut and contaminate the substrate when excreted in feces (Greenberg, 1971; Wallace, 1971; Greenberg, 1973; Graczyk et al., 2001b). In addition, the

prestomal teeth of the house fly mouthparts can contribute in their capacity to transmit bacteria, including pathogens. Previously, prestomal teeth of house flies was considered less damaging in comparison to other flies due to the smaller size, shape and more blunt apex as well as feeding behavior (Mahan'ko, 1973; Broce and Elzinga, 1984). The more recent report showed that the house fly prestomal teeth can be more damaging sometimes due to ≥ 2 vigourous movements when they are kept in one place. This teeth even can cut the epithelium conjunctiva about 30-50 μm deep which may allow the invasion of microorganisms into the host and successful pathogen transmission (Kovacs et al., 1990).

1.4. The house fly (*Musca domestica L.*) as a potential carrier for bacteria

Musca domestica (L.) belongs to the group of filth flies which poses tremendous health risk in public health as potential vector of microorganisms. Biologically and ecologically, the house fly habits and habitats make this insect a very effective mechanical vector for microbes. House flies have been regarded as mechanical or biological vectors of pathogenic bacteria including, *Salmonella* spp. (Greenberg, 1971), *Escherichia coli* O157:H7 (Kobayashi et al., 1999; Moriya et al., 1999; Sasaki et al., 2000; Alam and Zurek, 2004; Ahmad et al., 2007), *Proteus* spp., *Shigella* spp. (Greenberg, 1971), *Chlamydia* spp., *Campylobacter jejuni* (Shane et al., 1985), and *Vibrio cholerae* (Fotedar, 2001; Graczyk et al., 2001). They are also implicated in the transmission of other serious diseases such as anthrax, ophthalmia, typhoid fever, tuberculosis, cholera and infantile diarrhea (Scott and Lettig, 1962; Greenberg 1965; Keiding, 1986); protozoan infections such as amebic dysentery (Szostakowska et al., 2004); helminthic infections such as pinworms, roundworms, hookworms and tapeworms, as well as viral and rickettsial infections (Greenberg 1971; Gregorio, 1972; Greenberg, 1973; Graczyk et al., 2001a).

House flies were reported being involved in several disease outbreaks including *E. coli* O157:H7 (Sasaki et al., 2000) in Japan and *Vibrio cholerae* in India (Fotedar, 2001). House flies can also transmit eye diseases such as trachoma and epidemic conjunctivitis and infect wounds or skin leading to cutaneous diphtheria, mycoses, yaws and leprosy. House flies have been actively found with infectious *Chlamydia trachomatis* causing childhood blindness in children in endemic areas (Forsey et al., 1981; Emerson et al., 2000; Graczyk et al., 2001a).

Furthermore, house flies are now increasingly considered more than a simple mechanical vector based on several recent reports (Sasaki et al., 2000; Alam and Zurek, 2004, Macovei and Zurek, 2006; Petridis et al., 2006). A nationwide survey of Japan for the prevalence of enterohaemorrhagic *Escherichia coli* (EHEC) in house flies showed that flies (0.6%) carried the bacteria, and that almost all positive flies were collected from cowsheds and slaughterhouses. The authors also suggested that house flies were not simple mechanical vectors of EHEC, because the bacteria proliferated in the mouthparts and were excreted for at least 3 days after bacterial feeding after which the number of bacteria abruptly decreased in the gut (Sasaki et al., 2000). Recently, house flies have been shown to harbor antibiotic resistant bacteria that are an increasing public health concern. This study showed that house flies in urban fast-food restaurants commonly carried a large and genetically diverse population of enterococci with antibiotic resistance and virulence genes that were frequently expressed and likely carried on mobile genetic elements and therefore a food safety concern (Macovei and Zurek, 2006). Moreover, house flies have been reported as mechanical vector of nosocomial infections of multidrug-resistant bacteria in clinical settings (Fotedar et al., 1992; Rady et al., 1992).

1.5. Horizontal transfer of antibiotic resistance and virulence genes in digestive tract of house flies

The habitat of house flies harbors a diverse microbial community (Greenberg, 1973; Schmidtmann and Martin, 1992; Zurek et al., 2000; Graczyk et al., 2001; Moon, 2002). The rapid adaptation process of ingested microbes in the insect gut may involve gene transfer among different bacterial strains (Watanabe et al., 1998, Watanabe and Sato, 1998). Several studies suggested that the digestive tract of insects provides conditions conducive for horizontal transfer of antibiotic resistance genes on mobile genetic elements such as plasmids or transposons (Watanabe et al., 1998a; Watanabe et al., 1998b; Hinnebusch et al., 2002; Dillon and Dillon, 2004). Horizontal transfer of antibiotic resistance genes on plasmids has been shown in Collembola, *Folsomia candida* using *E. coli* in (Hoffman et al., 1998), *Erwinia herbicola* in the silk worm (Watanabe et al., 1998a; Watanabe et al., 1998b), and *Yersinia pestis* and *E. coli* in cat fleas (Hinnebusch et al., 2002). Recently, one study reported the horizontal transfer of antibiotic resistance genes among *E. coli* strains in the house fly digestive tract (Petridis et al., 2006). The authors reported the horizontal transfer of chloramphenicol resistance genes on a plasmid, or lysogenic, bacteriophage-born Shiga toxin gene *stx1* (bacteriophage H-19B::Ap1) from the donor to recipient *E. coli* strains. The study showed that the plasmid with the resistance gene was transferred in the fly midgut (10^{-2} / donor cell) and also in the crop (10^{-3} / donor cell). Transfer of the Shiga toxin gene *stx1* also occurred at a low frequency through bacteriophage transduction in the fly digestive tract. Plasmid transfer was detected in the fly gut after 1 hr exposure to the inocula. The authors suggested that the house fly digestive tract serves as an ideal spot for resistance gene transfer as well as rapid evolution of virulent strains (Petridis et al., 2006).

To our knowledge, there are no reports of horizontal transfer of antibiotic resistance genes or any other genes among Gram-positive bacteria in the house fly digestive tract.

2. Enterococci

2.1. Taxonomy and biology

Enterococci are Gram-positive cocci and a widely distributed group of bacteria in the phylum Firmicutes, class-Bacilli, order-Lactobacillales, family-Enterococcaceae, genus-Enterococcus (Schleifer and Kilpper-Balz, 1984). The cell arrangement is in single cells, pairs or short chains. They are facultative anaerobic, thermoduric, lactic acid bacteria. The optimum growth temperature of enterococci is 35^{o}C but they can grow in a wide temperature range from 10 to 45^{o}C at pH 9.6. Enterococci can grow in 6.5% NaCl and 40% bile salts and are capable to hydrolyze esculin (Sherman and Wing, 1935; Sherman and Wing, 1937; Moellering et al., 1999). Previously, enterococci were classified in the genus *Streptococcu*s as a group D-streptococci. In 1984, enterococci were separated to a different genus based on DNA-DNA re-association data, whole protein analysis, and 16S rRNA sequences which made them distinct enough from the *Streptococcus* genus (Schleifer and Kilpper-Balz, 1984; Moellering et al., 1999; Facklam et al., 2002). So far, 32 different species have been identified in the *Enterococcus* genus (http://www.bacterio.cict.fr/bacdico/ee/enterococcus.html, 2005).

2.2. Enterococci as a part of gut microbiota

Enterococci comprise the normal facultative anaerobic gut microbiota of animals, including humans (Mollering, 2000; Aarestrup et al., 2002; Simjee et al., 2006). They are found in mammals, birds, reptiles, and insects. The host range of enterococci is variable depending on

the species. In adult humans, enterococci represent only about 1.0% of intestinal microbiota (Sghir et al., 2000) but the population size is much more abundant in the infant gut (Stark and Lee, 1982). The fecal enterococcal concentration has been reported as high as 10^9 CFU/ g feces of 4 week old babies (Stark and Lee, 1982) whereas the average concentration range for adults is 10^5-10^7 CFU/ g feces (Jett et al., 1994). Enterococci can be also present in low numbers in oropharyngeal secretions, the urogenital tract, and skin. In humans, most common species are *E. faecalis* and *E. faecium*. *Enterococcus faecalis* is excreted in 90-100% and *E. faecium* in 25% of human feces (Moellering, 2000; Teixiera and Facklam, 2003). In farm animals, most frequently reported species are *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* (Devriese et al., 1987). Enterococci have also been reported from various insects including beetles, flies, termites, and bees (Martin and Mundt, 1972; Bauer et al., 2000). In insects, most common species were reported as *E. faecalis*, *E. faecium*, and *E. casseliflavus* (Fotedar et al., 1992; Sramova et al., 1992; Bauer et al., 2000; Macovei and Zurek, 2006). Enterococci were also found in various ecological habitats including soil, water, food, and plants, especially when contaminated with fecal material (Moellering, 2000; Giraffa, 2002).

2.3. Enterococci as opportunistic pathogens

For decades, enterococci were not considered as clinically significant (Murray and Weinstock, 1999). However, over the past decade, they have become the third most common causative agent of nosocomial infections in blood (Wisplinghoff et al., 2004) and the second most important causative agent of urinary tract infections and heart infections (Huycke et al., 1998; Bouza et al., 2001) in the United States. Overall, in the USA, 12.0% of the hospital acquired infections are caused by enterococci. These infections are sometimes life threatening

and difficult to treat due to the multidrug-resistance of these strains (McGowan et al., 2006; Comert et al., 2007; Hew, 2007). *Enterococcus faecalis* and *E. faecium* are the clinically most important species. Up to 80% clinical isolates are *E. faecalis* and 20% are *E. faecium* (Huycke et al., 1998; Reynolds et al., 2004; Coque et al., 2005). Although *E. faecalis* and *E. faecium* are the causative agents of most of the infections, the other species such as *E. gallinarum*, *E. avium,* and *E. durans* are also occasionally associated with human infections (Murray, 1990; Devriese and Pot, 1995; Tiexeira and Facklam, 2003; Mcgowan et al., 2006). Enterococci frequently also cause post surgical infections in humans (Malani et al., 2002).

Moreover, enterococci confer natural resistance to many antimicrobials that are used in clinical settings and also commonly acquire the resistance genes from other bacteria. Their ubiquitous nature contributes to the persistent infections and potential transfer of resistance genes to other microbes. Most importantly, since 1980s, enterococcal stains developed resistance to vancomycin (vancomycin is the last choice to treat the multidrug-resistant enterococci, recently quinupristin/dalfopristin also showed efficacy for VRE treatment) (Huycke et al., 1998; Winston et al., 2000). The emergence of vancomycin resistant enterococci (VRE) is a serious human health concern. According to the Center for Disease Control and Prevention (CDC), 28.5% of the nosocomial enterococcal infections in intensive care unit in USA are caused by VRE (vancomycin resistant enterococci (Lewis, 2002; NNIS report, 2004; Sherer et al., 2005; McGowan et al., 2006). After colonization in patients, 14% of VRE result into active infections within 15 days (Calfee et al., 2003). The risk factors for VRE colonization in hospital patients include inter-facility transfer and previous hospitalization record (Weinstein et al., 1996; Ostrowsky et al., 1999). VRE are also capable to transfer the *vanA* phenotype to more pathogenic bacteria such as *Staphylococcus aureus* which possess a serious threat in clinical settings

(Flannagan et al., 2003; Weigel et al., 2003). Most of the VRE isolates are multi-drug resistant and vancomycin resistance is most commonly found among *E. faecium* (Teixeira and Facklam, 2003). There is an increasing trend (20 fold from 1989 to 1993) of emergence of VRE which are responsible for nosocomial infections (Moellering, 2000; Mundy, 2000). Based on the clinical laboratory data, 52% *E. faecium* and 1.9% *E. faecalis* were vancomycin resistant in 1997 (Simjee et al., 2006). In the USA, increased mortality is reported for enterococcal endocarditis infections (Mc Donald et al., 2005). Severity of infections, age and prior antibiotic therapy are the common risk factors for mortality from enterococcal infections (Sood et al., 1998). Serious enterococcal infections are not easily treatable with one type of drug, so the drug of choice is a combination therapy of drugs (Herman et al., 1991).

2.4. Antibiotics and development of antibiotic resistance

Antibiotics are chemical compounds that act in a specific way to kill or inhibit the growth of bacteria. Some antibiotics are naturally produced by bacteria or fungi. Synthetic and semi-synthetic antibiotics are called antibacterials (Salyers and Whitt, 2005). Antibiotic resistance is the capability of bacteria and other microorganisms to resist the action of antibiotics. Multi-drug resistance has become a serious issue in clinical medicine. The main concern is the increase of resistance development in virulent bacterial strains and its correlation to the heavy use of antibiotics. The resistant bacteria are selected for by the selective pressure and by disseminating and sharing the resistance genes (Bogaard et al., 2000).

Bacteria develop resistance to antimicrobials in two main different ways: a) DNA mutation and b) acquisition of resistance genes from other bacteria by horizontal gene transfer. Bacteria can acquire resistance genes by three general mechanisms: transduction, transformation

and conjugation (Rice, 2000). In transduction process, DNA moves from one bacterium to another through a bacteriophage. Transformation mechanisms involve the intake of free DNA from the environment and integration into the host chromosome by DNA recombination (Dowson et al., 1989). Conjugation process occurs during cell to cell contact where DNA moves on a plasmid or transposon from a donor cell to a recipient cell (Swinfield et al., 1990; Dunny et al., 1995; Rice, 1998; Rice, 2000).

The rapid spread of antibiotic resistance is likely due to the heavy use of antibiotics in food animal productions and clinical medicine. Antibiotics are used for treatment and prophylaxis in clinical settings and animal productions. In the USA, antibiotics are used frequently as one of the feeding supplements to animal feed for growth promotion and to increase feed efficacy (Bogaard et al., 2000). The use of low level antibiotics (e.g. oxytetracycline, tylosin) in cattle feed and water are very important to producers to get efficient production, healthy livestock and higher longevity and productivity of food animals (Schroeder et al., 2002; Sprague, 2006; Sapkota et al., 2007). However, this heavy use of antibiotics has become a serious public health concern because the continuous practice of antibiotic use in food animals has lead to the rapid emergence, selection, and spread of resistant, commensal and potentially virulent bacterial strains. These resistant strains already have been reported from the food animals, in animal based food products, surrounding environmental samples (water, air, soil etc.) and also from farmers (Sørum et al., 2006; Sapkota et al., 2007,). Although the therapeutic and sub-therapeutic levels of antibiotics in animal feed is approved and regulated by FDA, no specific data collection system is available on the amounts that are used in animal feed. To close this data information gap several research groups have published estimates based on the USDA livestock production data and FDA antibiotic usage regulations. Approximately 60-80% of the

total antibiotics produced in the USA are used at sub-therapeutic levels in food animals (Mellon et al., 2007; Sapkota et al., 2007). Among these antibiotics, many compounds such as tetracyclines, macrolides, streptogramins and fluoroquinolones have analogue compounds that are also used in the clinical medicine (Turnidge, 2004; FDA, 2007; Sapkota et al., 2007). This poses a serious risk to public health due to the enormous selective pressure on environmental bacteria and consequently, the selection and spread of resistant strains to clinical settings.

European Union (EU) banned the antibiotic-based growth promoters in animal-feed in 1999 in attempt to reduce the prevalence of antibiotic resistant strains (Lester et al., 2006). In 2005, it was the first time the US Food and Drug Administration (FDA) banned a veterinary drug, enrofloxacin (Baytril), which had been used in poultry to treat respiratory disease (Turnidge, 2004). CDC reports showed that using this drug in poultry likely leads to an increase of ciprofloxacin resistant campylobacters which account 21% infections in humans per year (http:// www.cdc.org).

2.5. Antibiotic resistance of enterococci in humans and animals

Enterococci show intrinsic resistance to several categories of antibiotics, including low levels of aminoglycosides, beta-lactams (especially 3rd generation of cephalosporins), and quinolones (Ogier and Serror, 2008). Moreover, enterococci are capable to acquire frequent resistance and virulence genes through plasmids and transposons (Kak and Chow, 2002; Hew, 2006; Simjee et al., 2006). Over time, enterococci have become more important as pathogens and consequently placed under more antibiotic pressure for treatment of several serious infections resulting in greater selection of resistant strains.

Ampicillin belongs to the β-lactam group which acts by inhibiting the peptidoglycan synthesis. Enterococci show naturally, intrinsic resistance to β-lactam compounds because of their lower affinity to PBPs (penicillin binding proteins). However, the degree of resistance differs among different β-lactam agents for enterococci (Kak and Chow, 2002; Simjee et al., 2006). Aminoglycosides bind to the 16S rRNA of the 30S ribosomal subunit and inhibit the protein synthesis of bacteria. Enterococci show a very low level resistance to this class of drugs. Aminoglycosides act more effectively on enterococci in combination with penicillins or glycopeptides than alone (Kak and Chow, 2002). Streptomycin antibiotic falls into the class of aminoglycosides, which is produced as a secondary metabolite by *Streptomyces* strains. Since early 1940s, streptomycin had been used to treat several severe infections. Resistance mechanisms of enterococci to this class of antibiotics involve aminoglycoside modifying enzymes and resistance to the cell wall active agents (Fontana et al., 1996).

Glycopeptide antibiotics are also cell wall biosynthesis inhibitors of Gram-positive bacteria. They bind to D-Ala D-Ala and interfere with the cross-linking of the peptidoglycan. Vancomycin is one of the major one that is used in treating the severe multi-drug resistant Gram-positive infections. Enterococci confer resistance to glycopeptides by modifying the target by changing D-Ala-D-Ala presursor composition at one end which can be D-Ala-D-lactate or D-Ala-D-serine (Bugg et al., 1991). In enterococci, there are six gene clusters: *vanA, vanB, vanC, vanD, vanE, vanG*. Five of these are acquired resistance traits and one is intrinsic/natural resistance (*vanC*) (Gholizadeh and Courvalin, 2000; Kak and Chow, 2002). The specific resistance mechanisms are followed by a series of events, encoding the enzymes, sensing the presence of enzymes, making a resistant phenotype of peptidoglycan, and elimination of the

normal peptidoglycan precursors for prevention for further binding to cell wall (Kak and Chow, 2002)..

Macrolides inhibit protein synthesis of bacteria. Erythromycin falls in the macrolide group. It acts by binding to the 50S ribosome which blocks P site and inhibits peptidyl transfer and translocation (Weisblum, 1995; Jensen et al., 1999). Enterococci confer acquired resistance to this type of antibiotic by specific enzyme production which methylates an adenine residue in the 23S rRNA of the 50S ribosomal subunit, resulting in a lower binding efficacy to bacterial ribosomes (Jensen et al., 1999).

Chloramphenicol is also a protein synthesis inhibitor antibiotic. It acts by binding to the 50S ribosomes and blocks the peptidyl transferase reaction (Shaw, 1983). Chloramphenicol became the drug of choice for enterococcal infections due to resistance development to other drugs. But as it has been used very frequently, enterococci have developed resistance to this antibiotic as well (Kak and Chow, 2002). Chloramphenicol resistance is mediated by an acetyl transferase enzyme by which acetylation occurs in chloramphenicol hydroxyl molecule thus prevent ribosomal binding (Shaw, 1983).

Tetracycline also works as a protein synthesis inhibitor. It is a bacteriostatic drug and also inhibits protein synthesis by binding to the 30S ribosomal subunit and blocking the charged aminoacyl t-RNA binding (McMurray and Levy, 2000). This drug is commonly used in veterinary medicine (in food animals) as oxytetracycline and chlortetracycline. Enterococci show resistance by rapid efflux of the drug from the cell or by production of proteins that bind to the drug and protect bacterial ribosomes (Jones et al., 1998; Kak and Chow, 2002; FDA, 2007).

Ciprofloxacin belongs to the fluoroquinolone group and it is a synthetic compound. It shows bactericidal activity and inhibits DNA gyrase and topoisomerase and thus inhibits

bacterial DNA replication and specifically reduces DNA supercoiling. Enterococci confer resistance to ciprofloxacin by mutational change in the *parC* gene and the *gyrA* gene (Kanematsu et al., 1998; Kak and Chow, 2002).

2.6. Virulence determinants in enterococci

The majority of enterococci are commensals of animals and humans. This balanced relation can be changed depending on various predisposing factors and by some enterococcal strains acquiring virulence traits. Consequently, enterococci have become increasingly important as a serious nosocomial pathogen. Several studies showed that virulent lineages of enterococci are different from the normal flora populations (Willems et al., 2000; Kak and Chow, 2002). Enterococci can posses several factors which enable them to become more virulent. Cytolysin is a bacterial toxin which is expressed in ***E. faecalis*** and responsible for hemolytic and bactericidal activity (Hancock and Gilmore, 2000). A complex operon of eight genes encodes the production of cytolysin (Gilmore et al., 1990; Ike et al., 1990; Segarra et al., 1991; Gilmore et al., 1994; Coburn et al., 1999; Haas et al., 2002). The c*ylA* gene encodes the production of a serine protease similar to subtilisin which is secreted by general secretory pathway independently (Gilmore et al., 1990). The two structural subunits of cytolysin, $CylL_L$ and $CylL_S$ generate $CylL_L'$ and $CylL_S'$ which eventually generate the production of active toxin subunits $CylL_L''$ and $CylL_S''$ (Booth et al., 1984; Segarra et al., 1991). Another virulence factor of enterococci is gelatinase. It exhibits proteolytic activity and is capable to liquefy gelatin, lactoglobulin, fibrin, collagen, casein, and human endothelin (Makinen et al., 1989; Garcia de Fernando et al., 1991; Waters et al., 2003). Gelatinase is a zinc-mettaloprotease enzyme, encoded by ***gelE*** which is regulated by the ***fsr*** operon (Gilmore et al., 2002; Hancock and Perego, 2004). The role of *fsr* regulatory locus as a

two-component system in biofilm formation has been investigated. Studies with *E. faecalis* OG1RF showed that the complete *fsr* locus is needed for gelatinase production (Gilmore et al., 2002) and *gelE* inactivation can prevent biofilm formation in *E. faecalis* V583 (Hancock and Perego, 2004). Enterococcus protein, the aggregation substance, is a surface protein and encoded by pheromone responsive plasmid and plays an important role during conjugation and horizontal gene transfer (Clewell, 1993). Enterococcus surface protein, Esp acts as an adhesion protein and exhibits higher cell colonization in infections and also contributes to biofilm production (Shankar et al., 2001; Toledo-Arana et al., 2001; Hew et al., 2006).

2.7. Mobile genetic elements (plasmids and transposons)

Bacteriophages, plasmids, transposons, and insertion sequences carry DNA that can be transferred horizontally among different cells. Enterococci possess a great variety of plasmids and transposons some of which have been identified and studied. Plasmids and transposons of enterococci show similar characteristics to those of enterobacteria but some of them are very unique. This gene movement plays a very important role in spread of resistance and virulence traits. Enterococci are capable to transfer genes to more pathogenic bacteria such as *Staphylococcus aureus*, a potential human pathogen. Rapid emergence of MRSA (Methicillin resistant *Staphylococcus aureus*) and VRSA (vancomycin resistant *Staphylococcus aureus*) in hospitals is a very serious concern for public health. Recent report shows that the VRSA strain from US hospitals has acquired vancomycin resistance genes, eg. *vanA* from vancomycin resistant *E. faecalis* (Flannagan et al., 2003; Weigel et al., 2003; Sung and Lindsay, 2007). Vancomycin resistant *E. faecalis* V583 carries one of the highest proportions of mobile or acquired DNA among the known bacterial genomes. In V583, >25% of the whole genome

consists of mobile or acquired DNA. This mobile DNA includes three plasmids, seven integrated phage regions, 38 insertion elements, several conjugative and composite transposons, pathogenicity island, and integrated plasmid genes (Paulsen et al., 2003).

Enterococci have three types of self replicating plasmids: RCR (rolling circle replicating) plasmids, Inc18 plasmids and the most well studied pheromone responsive plasmids. The pheromone responsive plasmids are conserved to enterococci in terms of replication whereas the other two types of plasmids have the capacity to replicate non-specifically in Gram-positive bacteria.

Enterococci may acquire multi-drug resistance through different conjugation mechanisms. The conjugation mechanisms include pheromone responsive plasmid conjugation, non-pheromone responsive plasmid conjugation, and conjugative transposons. Pheromone responsive plasmid conjugation system is extensively studied in *E. faecalis* and it is restricted within the same species (Dunny et al., 1978; Buttaro et al., 2000; Hirt et al., 2005). In this conjugation process, pheromone oligopeptide and pheromone responsive plasmids are involved. *E. faecalis* recipient strains typically secrete peptide pheromone which induces the transfer of specific plasmids from the potential donor *E. faecalis* strains. The plasmid pCF10 specifically responds to cCF10 pheromone from the recipient cells. The plasmid pCF10 encodes the conjugation genes, cCF10 induces the expression of conjugative transfer genes, such as *prgB*. *prgB* then encodes the aggregation substance on the cell surface. This sticky protein causes cell-cell aggregation or clumping of cells. This clumping allows the transfer of specific pheromone responsive plasmid from donor to recipient strains (Olmsted et al., 1991; Bensing and Dunny, 1993; Murray, 1998; Buttaro et al., 2000). After the plasmid acquisition, the plasmid transfer stops but aggregation substance (Asc10) is present for long time on cell surface (Hirt et al.,

2005). This conjugation process is very efficient and was investigated in several *in vitro* and *in vivo* studies. In enterococci, five pheromone plasmids pAD1, pCF10, pPD1, pOB1 and pAM373 have been described, each of which responses to specific pheromones (Wirth, 1994).

Non-pheromone responsive conjugation mechanism involves plasmids which can replicate in a wide range of hosts. Enterococci are capable transfering resistance genes within and to different Gram-positive bacteria such as streptococci and staphylococci. However, plasmid transfer in this conjugation system is less efficient than pheromone responsive conjugation process (Clewell, 1981; Swinfield et al., 1990; Murray, 1998).

Enterococci have several distinct classes of transposons. Those are Tn3-family transposons, composite transposons, and conjugative transposons (Weaver et al., 2002). In this study, conjugative transposons, Tn*916* and Tn*916* /Tn*1545* family were screened from enterococcal isolates. Both Tn*916* and Tn*1545* act as important vector for *tetM*-mediated tetracycline resistance and they move from donor to recipient cells by intercellular contact. Tn*1545* can also confer erythromycin and kanamycin resistance (Weaver et al., 2002). Conjugative transposons are not self-replicative, so they may incorporate to a plasmid or the host chromosome and move within the same cell or to different cell via plasmids (Torres et al., 1991; Rice et al., 1992). Most of the conjugative transposons carry *tetM* which confers resistance to tetracycline and minocycline. Conjugative transposons are most efficient to transfer the resistance genes to many different bacteria due to their integration capacity into host chromosome or plasmids (Roberts, 1990a; Roberts, 1990b; Clewell, 1986; Murray, 1998).

Several *in vitro* and *in vivo* studies have been conducted to investigate the transfer efficiency of resistance genes/plasmids of among enterococci and from/to other genera. The plasmid with *tetM* and *ermB* has been transferred from *Lactobacillus plantarum* to *E. faecalis*

JH2-2 in the gastro-intestinal tract of gnotobiotic rats (Jacobsen et al., 2007). One report showed that plasmid transfer of *vanB* among *E. faecium* strains was more efficient *in vivo* experiments using a mice model than *in vitro* (Dahl et al., 2006). Recently, the transfer of *vanA* from *E. faecium* strain of animal origin to *E. faecium* isolate of human origin was reported in the human intestine. This *in vivo* study with human volunteers explored the serious risk factors of resistance genes acquisition as well as possible selection without any selective pressure (Lester et al., 2006). Moreover, a recent study reported the persistence of vancomycin resistance genes in VRE among environmental isolates. In Norway, after 3-8 years ban of glycopeptide avoparcin in food animals, glycopeptide resistant enterococci were characterized**.** A specific Tn*1546*-plasmid junction fragment has been found in 93.9% *E. faecium* isolates. One widespread plasmid mediated *vanA*-PRE25-PSK element was reported that maintains the resistance determinants without any selective pressure (Sørum et al., 2006).

The present study is focused on the role of house flies in the ecology and dissemination of antibiotic resistant and potentially virulent enterococci. House flies have been chosen for this study because of their high dispersal ability, vector potential, ecological habitat, and larval dependence on the bacterial community. The dependence of house flies on bacteria plays a vital role in the potential transmission of microbes. Enterococci were chosen as a bacterial model system because of their medical importance, frequent antibiotic resistance, great variety of mobile genetic elements, and their ubiquitous presence in the animal feces/manure and digestive tract of manure-borne insects. This research will be helpful for better understanding of the microbial ecology of house flies as well as the risk factors and molecular epidemiology and dissemination of antibiotic resistant enterococci in the environment

The specific objectives of this project:

1. **To assess the role of house flies in dissemination of antibiotic resistant and virulent enterococci**

2. **To determine the potential for horizontal gene transfer among *Enterococcus faecalis* strains in the house fly digestive tract**

3. **To evaluate the significance and survival of enterococci during the house fly development (egg to adult)**

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CHAPTER 2

Public health aspects of the house fly (*Musca domestica* L.) – *Enterococcus* spp. association: Characterization of enterococci from feces of feedlot cattle and American bison (*Bison bison* L.) and the digestive tract of associated house flies

ABSTRACT

Multi-drug resistance in bacteria has become a serious problem in clinical medicine. Though this remains a controversial issue, the connection between antibiotic resistance of the food animal origin and that of clinical isolates and community health has been suggested. Insects such as house flies that develop in decaying organic substrates including animal feces/manure may disseminate antibiotic resistant strains from rural to urban environments. House flies (FF) and cattle manure (FM) from a cattle feedlot (frequent use of antibiotics) as well as house flies (BF) and bison manure (BM) from the Konza Prairie Nature Preserve (no antibiotic use) were collected. Enterococci were quantified, identified, and characterized for selected antibiotic resistance and virulence factors and mobile genetic elements by polyphasic approach. Isolates from FF (n = 223), FM (n = 189), BF (n = 141), and BM (n = 167) were identified and characterized. Overall, the majority (89%) of samples were positive for enterococci at high concentrations (10^4 - 10^6 CFU/g of manure) and (10^5 - 10^6 CFU/fly) with the exception of BF (37.7% prevalence). My results show that phenotypically resistant enterococci to tetracycline and erythromycin were prevalent in the FM (Tet - Ery, 91% and 75%) and FF (Tet - Ery, 53% and 41%) which likely reflects the high antibiotic use in this environment. Although much lower, enterococcal resistance to tetracycline and erythromycin was also detected in the bison environment in BM (Tet-Ery, 3% and 19%) and BF (Tet-Ery, 18% and 9%). Overall, identified isolates represented *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus,* and *E. hirae.* The dominant species in cattle feedlot was *E. hirae* (91.9% in FM) and *E. faecalis* (35.7% in FF); *E. casseliflavus* was dominant (58.4 %) in BM, and *E. faecalis* (57.6 %) was most common in BF. Resistance genes (*tetM, tetS, tetO, ermB*) and the conjugative transposon Tn*916* were most commonly detected determinants from phenotypically resistant isolates from both environments.

In FM isolates, the highest multi-drug (six antibiotics) resistance was observed in *E. hirae* (1.9%) and 20% of *E. hirae* were resistant to four different antibiotics. *E. gallinarum* from FF (25.0%) showed multi-drug resistance to six antibiotics. *Enterococcus faecalis* (7.5%) and *E. faecium* (4.1%) were resistant to five antibiotics. There was no significant difference in tetracycline resistance profiles between FM and FF; however, a significant difference was observed in resistance profiles between BM and BF. Regardless of the samples and sites, by far the most dominant virulence gene was *gel*E of *E. faecalis* and it was phenotypically expressed (FM=100%, FF=82.5%, BM=73.6% and BF=75.5%). This study showed that antibiotic resistant enterococci are common in confined cattle production and house flies likely play an important role in the ecology of these bacteria in agricultural environments and potentially transmit antibiotic resistant strains to other environments.

INTRODUCTION

Antibiotic resistance and virulence in enterococci

Multi-drug resistance has become a serious issue in clinical medicine. The main concern is the increase of resistance development in virulent bacterial strains and the correlation to the increased use of antibiotics. The resistant bacteria are selected for by the selective pressure and by disseminating and sharing resistance genes (Bogaard et al., 2000). Bacteria can develop resistance to antimicrobials in two different ways: a) DNA mutation and b) acquisition of resistance genes from other bacteria by horizontal gene transfer. For example, ***Enterococcus faecium*** clonal complex (CC-1) clearly depicts the accumulation of adaptive changes in a bacterial population. ***E. faecium*** CC-1 acquired ampicillin-resistance determinants by the transposable element. Furthermore, in several instances, CC-1 acquired Tn*1546*, the transposable element that encodes vancomycin resistance (Baquero, 2004). Serious enterococcal infections are not easily treatable with one type of drug, so the choice is the combination therapy. For example, it has been reported that treatment of enterococcal carditis infections was ineffective with one antibiotic only (Herman et al., 1991).

Increased antibiotic resistance development and spread are likely due to a heavy use of antibiotics in food animal productions and clinical medicine. In domestic animals, antibiotics are used for treatment and prophylaxis. In the USA, antibiotics are also used continuously as one of the feeding supplements to animal feed for growth promotion and to increase feeding efficiency (Bogaard et al., 2000). The use of low level antibiotics (e.g. oxytetracycline, tylosin) in cattle feed and water are very important to producers for efficient production, healthy livestock and higher longevity and productivity of food animals (Schroeder et al., 2002; Sprague, 2006; Sapkota et al., 2007). However, this heavy use of antibiotics has become a serious public health

concern because the continuous practice of antibiotic use in food animals has lead to the rapid emergence and selection of resistant and potentially-virulent as well as commensal bacterial strains. These resistant bacterial strains already have been reported from food animals, in animal based food products, surrounding environmental samples (water, air, soil etc.) and also from farmers (Sørum et al., 2006; Sapkota et al., 2007).

Though the therapeutic and sub-therapeutic levels of antibiotics in animal feed are approved and regulated by Food and Drug Administration (FDA), no specific data collection systems are available on the amounts that are used in animal feed. To close these data information gap several research groups have published estimates based on the USDA livestock production data and FDA antibiotic usage regulations. Approximately 60 - 80% of the total antibiotics produced in the USA are used at sub-therapeutic levels in food animals (Mellon et al., 2007; Sapkota et al., 2007). Among these antibiotics, many compounds such as tetracyclines, macrolides, streptogramins and fluoroquinolones have analogue compounds that are also used in clinical medicine for humans (Turnidge, 2004; FDA, 2007; Sapkota et al., 2007). This poses a serious risk to public health due to the enormous selective pressure on environmental bacteria and consequently, the selection and potential spread of resistant strains to clinical settings and urban communities.

Animal and human feces are important reservoirs of diverse and numerous microbial communities that may contain human and animal pathogens. In addition, the development of antibiotic resistance among clinical isolates as well as commensal bacteria causes a great concern because of the potential dissemination and horizontal transfer of antibiotic resistance genes in the environment, primarily from the agricultural to urban environments.

Enterococci are gram positive cocci, diverse, ubiquitous group of lactic acid bacteria (Schleifer and Kilpper-Balz, 1984). The optimum growth temperature of enterococci is 35^{o}C but they can grow from 10 to 45^{o}C with a broad range of pH. They have the ability to grow in 6.5% NaCl and also hydrolyze esculin (esculin selective medium with 40% bile salts) (Teixeira and Facklam, 2003; Simjee et al., 2006). So far, 32 species have been identified in *Enterococcus* genus (http://www.bacterio.cict.fr/bacdico/ee/enterococcus.html, 2005).

Enterococci are most commonly found in the normal microbiota of GI tracts in animals and humans. They are also present in oropharyngeal secretions, the urogenital tract, and in lower numbers on the skin (Moellering, 2000; Tancock and Cook, 2002; Teixiera and Facklam, 2003, Simjee et al., 2006). They are frequently reported as the causative agent of nosocomial infections in the blood circulatory system, CNS (central nervous system), UTI (urinary tract infections) and heart (endocarditis). In the USA, 12% the hospital-acquired infections are caused by enterococci. These infections are sometimes life threatening and difficult to treat due to the multi-drug resistance (McGowan et al., 2006; Comert et al., 2007; Hew, 2007). Among all species, *E. faecalis* and *E. faecium* are clinically the most important. Although *E. faecalis* and *E. faecium* are the causative agents of most of the infections, other species such as *E. gallinarum*, *E. avium* and *E. durans* are also infrequently found in human infections (Murray, 1990; Devriese and Pot, 1995; Tiexeira and Facklam, 2003; Mcgowan et al., 2006; Simjee et al. 2006). Moreover, enterococci confer natural resistance to many antimicrobials (e.g. low levels of aminoglycosides) that are used in clinical settings and also commonly acquire resistance genes from other bacteria by horizontal gene transfer through plasmids and transposons (Kak and Chow, 2002; Hew, 2006; Simjee et al., 2006). Their ubiquitous nature makes them more likely to transfer the resistance genes to other microbes. Most importantly, since the 1980s, *E. faecium* and *E. faecalis* acquired

resistance to vancomycin, (vancomycin, quinopristin-dalfopristin are the last choice antibiotics to treat drug-resistant infections). According to Center for Disease Control and Prevention (CDC), 28.5% of the nosocomial enterococcal infections in intensive care unit in USA are VRE (vancomycin resistant enterococci) (Lewis, 2002; Sherer et al., 2005; McGowan et al., 2006).

Several reports support the suggestion that more virulent lineages of enterococci are related with the clinical isolates and they are different from the normal enterococcal environmental populations (Gilmore et al., 2002). Enterococci posses several virulence factors that are associated more frequently with clinical isolates (Gilmore et al., 2002; Hew et al., 2006). Cytolysin is a bacterial toxin which is expressed in *E. faecalis* and responsible for hemolytic and bactericidal activity (Hancock and Gilmore, 2000). A complex operon of eight genes encodes the production of cytolysin. The *cylA* gene encodes the serine protease similar to subtilisin. Another virulence factor of enterococci is the enzyme gelatinase. Gelatinase is a metaloprotease regulated by the *fsr* operon. It exhibits proteolytic activity and has the ability to hydrolyze gelatin, lactoglobulin, collagen and other proteins and it is involved in biofilm formation (Gilmore et al., 2002; Hancock and Perego, 2004; Hew et al., 2006). The aggregation substance is a surface protein encoded by pheromone responsive plasmids and it plays a role during conjugation and horizontal gene transfer. Enterococcus surface protein *esp* acts as an adherent and exhibits higher cell colonization in infections as well as contributes to biofilm production (Shankar et al., 2001; Toledo-Arana et al., 2001; Hew et al., 2006).

Bacteriophage, plasmids, transposons/insertion sequences carry DNA that is conserved and can be transferred among bacteria as well as the two prokaryotic domains (Weaver et al., 2002). Enterococci posses great variety of plasmids and transposons, many of which have already been identified and thoroughly studied. Although plasmids and transposons of

enterococci show similar characteristics to those of enterobacteria; some of those are very unique. Plasmids and transposons are responsible for resistance gene mobilization within and between bacterial species and genera. This gene movement plays a very important role in evolution of resistance and pathogenic and virulent strains. Enterococci are capable of transfer these genes to more pathogenic bacterial strains such as ***Staphylococcus aureus***, a potential human pathogen (Weigel et al., 2001). Rapid emergence of MRSA (methicillin resistant ***Staphylococcus aureus***) and VRSA (vancomycin resistant ***Staphylococcus aureus***) in hospitals is a very serious concern for public health. Recent reports show that VRSA strains from US hospitals have acquired the resistance gene, ***vanA*** from vancomycin resistant ***E. faecalis*** (Weigel et al., 2001). It is reported that ***E. faecalis*** pAM830 transferred the ***vanA*** on Tn***1546*** through donor plasmid to ***S. aureus*** though this plasmid was not stable (Sung et al., 2007). Clinical isolate V583 (vancomycin resistant ***E. faecalis***) carries one of the highest proportions of mobile or acquired DNA among the known bacterial genomes. In V583, more than 25% of the whole genome consists of mobile or acquired DNA (Paulsen et al., 2003). This mobile DNA includes the ***vanB*** gene on a transposon, three plasmids, seven integrated phage regions, 38 insertion elements, several conjugative and composite transposons, pathogenicity island, and integrated plasmid genes. This large portion of portable DNA might be a great contributor in spreading virulence and antibiotic resistance (Paulsen et al., 2003).

In this study, conjugative transposon Tn***916*** and Tn***1545*** with a broad host range were screened in our isolates. Both Tn***916*** and Tn***1545*** act as important vectors for carrying ***tetM***-mediated tetracycline resistance and they move from donor to recipient cells by conjugation. Tn***1545*** can confer erythromycin and kanamycin resistance. Both Tn***1545*** and Tn***916*** share the same structural ending of a common ancestor (Weaver et al., 2002).

*Role of the house fly (*Musca domestica L.*) as a potential vector for bacteria*

Musca domestica L. belongs to the group of muscoid flies that posses tremendous health risk in public health as potential vector of microorganisms (Grazcyk et al., 2001). This occurs in all continents except Antarctica. They are non-biting insects and have sponging type of mouthparts. Larvae feed on a moist food rich in organic matter (Moon, 2002). Although they are attracted to a variety of food material, House flies have sponging mouthparts which allow them to ingest only liquid food whereas solid foods are liquefied by means of regurgitated saliva. House flies require an active microbial community for larval development (Zurek et al., 2000). Therefore, immatures can be found in a wide variety of decaying organic substrates rich in microbes. They oviposit and develop mainly in decaying organic matter, human garbage dumps, open privies, animal and human excretion, animal bedding, soil bedding, poultry litter and also in wastes around food and vegetable processing plants which has a diverse and active microbial community (Graczyk et al., 2001; Moon, 2002). House flies can breed outdoors throughout the year in tropical countries but not in winter in temperate regions. Larval developmental habitat, unrestricted movement of adults, mode of feeding, and attraction to human food and drinks make this insect an ideal vector for bacteria originating from feces, manure, and other decaying substrates (Grazcyk et al., 2001). In addition, the house fly life cycle takes only 14 days to complete in summer and they are multivoltine. Female house flies longevity is 15-25 days and they lay 5-6 batches of eggs in their lifetime (Moon, 2002). House flies resume the reproductive cycle inside cattle barns and poultry houses in winter time. Adult house flies aggregate around garbage, compost piles, and enter buildings in search for food and warmth (Moon, 2002).

House flies can carry bacteria in their gut or in other body parts or body surfaces (Moon, 2002). They have been regarded as mechanical vectors of pathogenic bacteria including

Salmonella, Escherichia, Proteus, Shigella, Chlamydia, and Campylobacter, Vibrio cholerae (Graczyk et al., 2001). They may be also actively involved in transmission of other serious infections such as anthrax, ophthalmia, typhoid fever, tuberculosis, cholera and infantile diarrhea (Scott and Lettig, 1962; Greenberg, 1965; Keiding, 1986). They are also associated with protozoan infections such as amebic dysentery; helminthic infections such as pinworms, roundworms, hookworms and tapeworms (Greenberg, 1971; Gregorio, 1972; Greenberg, 1973; Graczyk et al., 2001), as well as viral and rickettsial infections (Graczyk et al., 2001; Gorham and Zurek, 2006). House flies are potential carriers of *Escherichia coli* O157:H7 and also involved in several disease outbreaks such as *Escherichia coli* O157:H7 in Japan and *Vibrio cholerae* in India (Kobayashi et al., 1999; Fotedar et al., 2001; Petridis et al., 2006). Flies can also transmit pathogens of eye diseases such as trachoma and epidemic conjunctivitis, and infect wounds or skin with pathogens of cutaneous diphtheria, mycoses, yaws and leprosy. Fly transmitted trachoma alone causes 6 million cases of childhood blindness each year (Graczyk et al., 2001). House flies are highly mobile and they can travel as far as 20 miles so they can spread this pathogens also to the surrounding environments (Moon, 2001). It is not surprising that they are involved in transmission of so many serious and widespread diseases. They can potentially contaminate any substrate by the unique regurgitation habit and/or fecal excretion. The pathogen transmission mechanism involves feeding on fecal material with pathogens, ingestion and regurgitation of pathogen contaminated digestive fluids or fecal excreta on human food (Osato et al. 1998; Graczyk et al., 2001).

House flies were chosen in this study because of their larval development in animal feces/manure, high dispersal ability of adults (Broce, 1993) and vector capacity for bacteria. The dependence of house fly larval development on bacterial communities indicates a close

evolutionary association. The physiological association between house flies and bacteria plays a vital role in potential transmission of microbes. Also to have a complete understanding of pathogen or microbe dissemination, house flies need to be studied with microorganisms in an ecological context. In this study, the role of house flies in the ecology of enterococci and associated antibiotic resistance and virulence genes from animal manure was assessed.

Rationale:

This research should be helpful in better understanding the microbial ecology of house flies as well as the role of these flies in the ecology of antibiotic resistance and virulence genes in agricultural and nature preserve environments.

HYPOTHESIS:

House flies act as vectors of antibiotic resistance and virulence genes associated with enterococci originating from manure of food animals

OBJECTIVES:

The general objective of this research was to assess the role of house flies in the ecology of enterococci and associated antibiotic resistance and virulence genes by comparing enterococci population of house flies from a cattle feedlot and an American bison pasture and corresponding animal feces.

Specific approach

1. Isolation, quantification, identification, and characterization of enterococci from the feces of animals frequently exposed to antibiotics (feedlot cattle) and animals with no exposure to antibiotics (American bison) and from the associated house flies.
2. Assessment of the antibiotic resistance profile of enterococci by phenotypic analysis
3. Determination of the prevalence and diversity of tetracycline and erythromycin resistance genes of identified isolates of enterococci.
4. Assessment of the prevalence of virulence factors of enterococci including gelatinase, hemolysis, and aggregation substance by phenotype.
5. Evaluation of the prevalence of enterococcal virulence genes including *cylA* (hemolysis activity), *gelE* (proteolytic activity), *asa1* (aggregation substances) and *esp* (enterococcus surface protein).

   Assessment of the prevalence of mobile genetic elements with a broad host range: Tn*1545/916* family and Tn*916*.

MATERIALS AND METHODS

Selection of sites. Sites for sample collection were determined based on the status of antibiotic use on animals. All samples were collected from two sites. One was an experimental cattle feedlot of the K-state Agricultural Experimental Station located approximately 2 km northeast of Manhattan, KS. Antibiotics (monensin, tylosin phosphate, chlortetracycline hydrochloride, oxytetracycline hydrochloride, tiamulin) (Food and Drug Administration, 2007) are frequently used in feedlot cattle. The other site was the tall grass prairie on Konza Prairie Nature Preserve Biological Station. This is a 3487-hectare area in the Flint hills of northeastern

Kansas located approximately 13 km south of Manhattan, KS. Konza prairie inhabitants include large grazers such as American bison with no antibiotic exposure (www.konza.ksu.edu).

Collection of samples. Fresh (<2-5 hr) cattle fecal samples (n = 110) were collected from the cattle feedlot into sterile plastic bags using sterile tongue compressors. Associated house flies (n = 124) were collected from feed bunks and cattle pens. Bison manure samples (n = 23) were collected directly manually from the rectum of a group of two year old American bison (Konza Prairie Biological Station) during the annual spring round up for vaccination and weight determination. House flies (n = 114) were collected from fences of the bison either by sweep net or sticky traps. Sticky traps were routinely checked in every other day. All samples were immediately placed in the cooler with ice bags and transported to the laboratory.

Isolation of enterococci. All samples were processed immediately after the arrival to the laboratory. One gram of cattle and bison fecal samples was homogenized in 10 ml of PBS (Phosphate Buffer Saline, pH 7.0; MP Biomedicals, USA) and serially diluted. House flies from both sites were surface sterilized with sodium hypochlorite and ethanol (Zurek et al., 2000) and homogenized individually in 1 ml PBS. All manure and fly samples were diluted into two 10-fold serial dilutions and drop plated on m-Enterococcus agar (Difco, Franklin Lakes, NJ). Plates were incubated at 37^{o}C for 24 - 48 hrs. After incubation, the concentration of enterococci was determined by colony counting method (CFU count) and 3-5 dark purple colored colonies (presumptive) of *Enterococcus* genus were picked from each sample for confirmation tests. Each colony was streaked on TSBA (Trypticase Soy Broth Agar, Difco) to obtain the pure culture and processed for confirmatory biochemical analysis. Each strain was cultured in enterococcosel

broth (Difco) in 96-well plates to confirm the *Enterococcus* genus by the esculin hydrolysis test. Also, the growth of isolates was confirmed in Trypticase Soy Broth (Difco) with 6.5% NaCl at 44^{o}C (Facklam et al., 2002). All positive isolates were stabbed into TSBA (0.3% agar) in 2.0 ml vials and stocked at room temperature.

Identification of enterococcal species. *Enterococcus* species were determined by multiplex and single PCR. Four different species including *E. faecalis, E. faecium, E. casseliflavus* and *E. gallinarum* were identified by multiplex PCR (Kariyama et al., 2000; Elsayed et al., 2001). *E. mundtii* ATCC 43186 was used as a negative control. *E. hirae* was identified by single PCR and *E. hirae* ATCC 8043 was used as a positive control. Only 11 isolates of cattle manure from a feedlot were identified by multiplex PCR. To identify the isolates from cattle manure, the *sodA* gene (superoxide dismutase) was amplified by PCR and then sequenced and confirmed by BLAST search in the NCBI GenBank database (Poyart et al., 2000). All identified isolates were processed for further characterization.

Antibiotic susceptibility by phenotype. All identified isolates from bison manure, cattle manure, and house flies were assessed for antibiotic susceptibility test. This screening was done by disk diffusion technique using the Mueller-Hinton Agar (Difco) and six different antibiotics, tetracycline (30 μg), erythromycin (15 μg), vancomycin (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), and ampicillin (10 μg). Susceptibility to aminoglycosides such as streptomycin (2000 μg/ml) and kanamycin (2000 μg/ml) was evaluated by the agar dilution method using TSBA plates. The Clinical and Laboratory Standards Institute protocols were used as standards for these procedures (Clinical and Laboratory Standards Institute, 2000).

Virulence factors (Gelatinase activity, aggregation substances and hemolysis by phenotype). Gelatinase activity was determined by using Todd Hewitt Broth (THB) Agar plates with 1.5% dry milk incubated at 37^{o}C for 24 hrs. After the incubation period, the clearance zone was measured to assess gelatinase activity.

Cytolysin gene expression was evaluated by streaking the isolates on Columbia blood agar plates with 5% human blood and incubated at 37^{o}C for 24 hrs. Hemolytic activity was assessed by measuring the partial or total clearing zone around colonies. Total clearance zone was determined as the β-hemolysis or complete hemolysis and recorded positive for cytolysin gene expression.

Enterococcal aggregation substance was screened phenotypically for all *E. faecalis* strains by the clumping assay (Dunny et al., 1978). In this assay, *E. faecalis* JH2-2 was used for cCF10 peptide formation. THB was used to grow *E. faecalis* JH2-2 and incubated at 37°C for 18 hrs. The pheromone peptide in the supernatant was collected by centrifuging (10,000 rcf for 10 min) and sterilized by autoclaving for 15 min. *E. faecalis* isolates were cultured in THB broth for 18 hrs at 37^{o}C, then 1 ml *E. faecalis* JH2-2 supernatant was added to each culture and incubated at 37°C overnight in a shaker incubator. After the incubation period, isolates were considered positive if clumping or aggregation of cells was observed by naked eye or under a microscope. *E. faecalis* OG1RF (pCF10) was used as positive control with every batch of isolates (Dunny et al., 1978).

Antibiotic resistance genes and virulence genes. All identified isolates from bison and cattle manure and house flies were screened for tetracycline and erythromycin resistance genes by multiplex and single polymerase chain reaction (PCR). Tetracycline resistance genes were

divided into three groups. Group I included *tetA*, *tetC*, and *tetQ* genes. Group II included the *tetM, tetS, tetK,* and *tetO* genes. The protocols were followed as described previously (Ng et al., 2001; Turnidge et al., 2004). Group III included the *tetW* gene (Aminov et al., 2001) and group IV erythromycin gene, *ermB* (Sutcliffe et al., 1996) which were detected by single PCR.

Four virulence genes were screened for: *gelE* (gelatinase activity), *asa1* (aggregation substances), *cylA* (cytolysin, hemolytic activity), and *esp* (enterococcus surface protein) and identified by multiplex PCR (Vankerckhoven et al., 2004).

*Mobile genetic elements (*Tn*916 and* Tn*1545/916 family).* Integrase gene (*int*) was used for detection of the Tn*916/*Tn*1545* conjugative transposon family (Doherty et al., 2000; Gevers et al., 2003) which frequently carry the tetracycline resistance genes *tetM*. The ORF13 gene was used to detect the transposon Tn*916* (Andrews et al., 2004). *E. faecalis* OG1RF (pCF10) was used as a positive control for both PCR.

Statistical analysis. The statistical analysis was done by using Chi-square analysis of contingency tables test ($p<0.05$) and Fisher's exact test ($p<0.05$) with the SAS statistical package (SAS institute, 2003).

RESULTS

Prevalence, quantification, and identification of enterococci in fecal samples and house flies from Konza Prairie and cattle feedlot

The prevalence of enterococci was high in most of the samples including, BM (100%), FM (77.2%), FF (96.8%) with the exception of BF where prevalence was only 37.7% (Table 1).

The enterococcal concentration in positive samples was high across all sampled sites and ranged from $5.1\pm 3.8x10^6$ CFU per g (BM) to $1.4 \pm 0.5x10^5$ CFU per BF (Table 1). A subset of isolates from each site including BM (n=176), BF (n=141), FM (n=189) and FF (n=223) was selected for identification and further characterization. Multiplex or single PCR resulted in identification of enterococcal isolates in range from 74.8% (BM) to 50.2% (FF) (Table 1).

The majority of identified isolates from BM was represented by *E. casseliflavus* (58.4%), followed by *E. faecalis* (15.2%), *E. hirae* (12.0%), and *E. faecium* (8.0%). In contrast, BF carried more *E. faecalis* (57.6%) followed by *E. casseliflavus* (42.3%) and *E. hirae* (0.9%). *E. gallinarum* was not detected in samples from Konza prairie (Table 1).

The dominant enterococcal species in FM was *E. hirae* (91.9%), followed by *E. casseliflavus* (6.3%), *E. faecalis* and *E. faecium* (both 0.8%). The diversity of enterococci in FF was more evenly distributed among *E. faecalis* (35.7%), *E. faecium* (21.4%), *E. gallinarum* (19.6%), and *E. casseliflavus* (17.9%) (Table 1).

Prevalence of each species was significantly different ($P<0.0001$) within and between two different sites except *E. casseliflavus* in BM and BF ($P<0.99$) and *E. faecalis* in BF and FF ($P<0.99$) (Table 1).

Phenotypic antibiotic resistance

All identified isolates were tested for antibiotic susceptibility to eight antibiotics: tetracycline (TE), erythromycin (E), vancomycin (VA), kanamycin (K), streptomycin (S), ciprofloxacin (CIP), chloramphenicol (C) and ampicillin (AM). The main emphasis was to test tetracycline and erythromycin susceptibility as these two antimicrobials are widely used in food animals for growth promotion. In BM, TE resistance was found at a low frequency and only in *E. casseliflavus* (2.7%) and in *E. hirae* (13.3%) (Fig. 3). *E. faecalis* (36.8%), *E. faecium* (70.0%), *E. hirae* (26.7%) and *E. casseliflavus* (8.2%) from BM showed resistance to erythromycin. The majority of isolates from BM exhibited susceptibility to ciprofloxacin with the exception of *E. casseliflavus* which is intrinsically resistant to ciprofloxacin (Figure 3). *E. faecalis* (57.9%) isolates showed resistance to kanamycin. Streptomycin resistance was not found among BM isolates (Fig. 3). Fifteen percent isolates of *E. faecalis* were resistant to three different drugs and 10% *E. faecium* showed multi-drug resistance for four antibiotics.

There was a similarity in ciprofloxacin resistance between BM and BF isolates. In BF samples, the majority of *E. faecalis* (60%) and *E. casseliflavus* (69.23%) isolates were resistant to ciprofloxacin. *E. faecalis* (20.8%) as well as *E. casseliflavus* (7.7%) showed resistance to vancomycin. No kanamycin resistance was detected in BF isolates. In BF, *E. faecalis* showed tetracycline (24.5%) and erythromycin (5.7%) resistance whereas *E. casseliflavus* had resistance 10.3% for tetracycline and 15.4% for erythromycin (Fig. 4). Three isolates of *E. faecalis* showed resistance to three different antibiotics.

Overall, there was a significant difference in prevalence of antibiotic resistance between BM and BF isolates. There was no significant difference between BM and BF isolates for

ampicillin (P = 0.09), chloramphenicol (P = 0.76) and ciprofloxacin (P = 0.99) resistance profiles.

In contrast, a very high antibiotic resistance was observed in FM isolates for tetracycline and erythromycin (Fig. 1). The majority of *E. hirae* were resistant to tetracycline (94.2%) and erythromycin (77.7%) followed by chloramphenicol (55.3%), ciprofloxacin (43.7%), and a small portion (6.8%) was also resistant to vancomycin (Fig. 1). No streptomycin, kanamycin, or ampicillin resistance was found in FM. One *E. faecalis* from FM exhibited resistance to tetracycline, erythromycin, ciprofloxacin and chloramphenicol. Forty two percent of *E. casseliflavus* showed resistance to tetracycline, erythromycin and ciprofloxacin. In FM samples, the highest multi-drug resistance was observed in *E. hirae* (1.9%) for six different antibiotics and 20% of *E. hirae* isolates were resistant to four different antibiotics.

Among all samples, most frequent resistance to various antibiotics was detected in FF isolates (Fig. 2). Tetracycline resistance was most highly prevalent in *E. faecium* (60.9%), *E. faecalis* (55.0%), and *E. gallinarum* (52.4%), followed by erythromycin resistance in *E. faecium* (47.6%) and *E. faecalis* (40.0%). *E. casseliflavus and E. hirae* also expressed frequent tetracycline (40.0% and 50.0%, respectively) and erythromycin resistance (50.0% and 12.5%, respectively). High ciprofloxacin resistance was observed in *E. casseliflavus* (80.0%), *E. faecium* (60.9%), *E. gallinarum* (47.6%), *E. faecalis* (32.5%), and *E. hirae* (12.5%). *E. gallinarum* (25%) showed multi-drug resistance to six antibiotics. *E. faecalis* (7.5%) and *E. faecium* (4.1%) were resistant to five antibiotics. At least two different species exhibited resistance to at least two antibiotics.

There was no significant difference observed in different antibiotic resistance profiles between FM and FF isolates except ampicillin (P=0.002) and kanamycin (P=0.001).

Gelatinase activity, aggregation substances and hemolysis by phenotype

All identified isolates from BM, BF, FM and FF were tested for gelatinase activity. Hemolysin (cytolysin acivity) test was done on all isolates using human blood agar. Clumping assay to determine the activity of aggregation substances was conducted on *E. faecalis.*

In BM isolates, all *E. faecalis* showed proteolytic (gelatinase) activity (Fig. 3). Also, *E. faecium* (70%), *E. hirae* (60.0%), and *E. casseliflavus* (15.1%) exhibited proteolytic activity on 1.5% milk agar plates. None of the *E. faecalis* isolates from BM showed aggregation in the clumping assay (Fig. 3). Hemolytic activity on human blood was found only in *E. faecium* isolates (10%) from BM (Fig. 3).

Among BF isolates, 79.2% of *E. faecalis* were positive for gelatinase activity (Fig. 4). None of *E. casseliflavus* was positive for the gelatinase phenotype. Only 5.6% of *E. faecalis* isolated from BF showed aggregation in the clumping assay (Fig. 4). On human blood, 39.6% of *E. faecalis* were hemolytic (Fig. 4).

Among the feedlot site samples, none of the FM isolates were positive for proteolytic activity (Fig. 1). None of the *E. faecalis* isolates from FM were positive for the aggregation substance (Fig. 1). Only 14.3% *E. casseliflavus* showed hemolytic activity on human blood (Fig.1).

In FF samples, 72.5% *E. faecalis* showed proteolytic activity (Fig. 2) which was followed by *E. casseliflavus* (30.0%), *E. gallinarum* (13.6%) and *E. faecium* (12.5%). Aggregation substance was detected in 5% *E. faecalis* isolates from FF (Fig. 2). The highest number of *E. faecalis* isolates (45.0%) from FF was hemolytic on human blood, followed by *E. faecium* (33.3%), *E. casseliflavus* (25%) and *E. hirae* (12.5%) (Fig. 2).

There was no significant difference observed of hemolytic activity (*P*=1.0) and gelatinase activity (*P*=0.68) between BM and FM isolates.

Screening of antibiotic resistance genes, virulence genes, mobile genetic elements (Tn916 and Tn1545/916 family) and correlation of resistance and virulence genes with phenotypic expression

Eight *tet* genes were screened in this study along with *ermB* and Tn*916* and Tn*1545/916* family. In BM isolates, the most common combination observed was *tetM* with *ermB* and Tn*916* for *E. hirae* (13.3%) in which 100% of isolates with the *ermB* gene were phenotypically expressed (Table 3). Among *E. faecalis*, several isolates (31.5%) were positive for *tetK* without any phenotypic expression. Multiple *tet* genes (*tetM, tetK*) and Tn*916* were present in 5.2% *E. faecalis* isolates. Thirty percent of *E. faecium* isolates either carried *tetM* or *tetK* with no phenotypic expression. *E. casseliflavus* commonly carried *tetM* and 1.4% isolates were positive *tetM* with Tn*916* with phenotypic expression of tetracycline resistance.

In BF samples, *tetM* was also the most commonly observed resistance determinant. Among *E. hirae*, 17.5% isolates were found to have the combination of *tetM* (phenotypic expression 94.4%) with *ermB* (phenotypic expression 72.2%) and Tn*916*. (Table 3). Another frequent combination (17.5%) observed in *E. hirae* was *tetO* (100% expressed) with *ermB* (88.9% expressed). One isolate of *E. faecalis* had *tetO* and *ermB* with 100% expression. One *E. faecium* had *tetK* which was phenotypically expressed (Table 2). *E. faecalis* (2.0%) along with *ermB* and Tn*916* exhibited 100% phenotypic expression. Most frequent *tet* resistance gene was *tetO* in *E. casseliflavus* (15.4%) (Table 3).

FF isolates represented the most diverse group with the highest frequency of resistance and virulence factors (Table 2). *E. faecalis* (22.5%) had *tetM* as the most common *tet* resistance gene (100% phenotypic expression) with Tn*916* and Tn*1545/916* family. In *E. faecalis*, all (100%) *tet* and *ermB* resistance genes were phenotypically expressed. *TetM* also was the most common resistance determinant in *E. faecium* (33.3% with 87.5% phenotypic expression) (Table 3).

Among the virulence factors, *gelE* was the most frequently found virulence gene in *E. faecalis* from BM (73.6% with 100% gelatinase activity) and BF (75.5% with 87.5% gelatinase activity) (Table 5). *E. faecalis* (5.6%) was positive for *gelE* (100% phenotypic expression) and *asa1* (33.3% phenotypically expressed).

Most of the FF isolates carried one or more virulence determinants. *E. faecalis* isolates were positive for *gelE*. *E. faecalis* carried the most common virulence determinant, *gelE* (100% in FM and 82.5% in FF) with phenotypic activity (100% in FM and 72.7% in FF). The *cylA* gene was found in *E. casseliflavus* (14.3%) and *E. hirae* (1.9%) from FM isolates with no phenotypic expression. In FF, 2.5% isolates of *E. faecalis*, 4.7% isolates of *E. faecium* and 5% isolates of *E. casseliflavus* also were positive for *gelE* with *asa1* and *esp* (Table 4).

DISCUSSION

Enterococci are common members of the normal microbiota of the intestine of humans and other animals (Mollering, 2000; Aarestrup et al., 2002; Simjee et al., 2006). However, some enterococcal strains, especially of *E. faecalis* and *E. faecium* are opportunistic human pathogens causing for example endocarditic, urinary tract infections, and peritonitis (Huycke et al., 1998; Mollering, 2000; Reynolds et al., 2004; Coque et al., 2005). In addition, there is a growing concern about the development of multi-drug resistance in enterococci. Twenty nine percent of nosocomial infections in USA hospitals are caused by multi-drug resistant enterococci which includes in some cases resistance to vancomycin (Kummerer, 2003; Sherer et al., 2005; Heuer, 2006; Simjee et al., 2006). Furthermore, enterococci have the ability to acquire and transfer the resistance genes including vancomycin resistance genes to other strains of more pathogenic bacteria, such as *Staphylococcus aureus* (Weigel et al., 2003; Levy and Marshall, 2004; Lester et al., 2006).

In the USA, approximately 70% of the total produced antibiotics are used in livestock production (Mellon et al., 2007; Sapkota et al., 2007). The heavy use of antibiotics in livestock production contributes to resistance development and a growing reservoir of resistant enterococcal population (Simjee et al., 2006). This raises the debate on several important public health issues. Most of the antibiotics that are used in food animals are analogues of drugs that are used in human thus might confer the resistance to human drugs (with some exceptions including ionophones, quinoxalines, and avilamycins). The relationship between antibiotic use and the development and spread of antibiotic resistance has been studied extensively in clinical as well as environmental isolates (Kummerer et al., 2003; Turnidge et al., 2004; Macovei and Zurek, 2006; Simjee et al. 2006; Sørum et al., 2006).

In this study, samples were collected from two different settings to evaluate the prevalence and diversity of antibiotic resistance and virulence factors associated with enterococci and to assess the potential role of house flies as vectors for these enterococcal strains. House flies can only develop in decaying organic substrates such as animal manure with an active microbial community (Zurek et al., 2000; Graczyk et al., 2001; Moon et al., 2001; Moon, 2002) and adult flies commonly carry in their digestive tract bacteria originating from the larval habitat (manure, feces, compost) (Zurek et al., 2000; Graczyk et al. 2001). It has been reported that house flies carry enterococci in the alimentary tract in high concentrations (Macovei and Zurek, 2006). The developmental habitat, unrestricted movement of adult flies (Broce, 1993) and their feeding mode (regurgitation) make house flies a likely candidate as a vector of bacteria originating from decaying organic substrates, including feces and manure.

Feedlot cattle manure (FM) and house flies (FF) were obtained from a cattle feedlot where antibiotics are used as promoters and for prophylaxis and treatment. American bison manure (BM) and house flies (BF) were collected from the Konza nature preserve where no antibiotics are used. Therefore, BM and BF are considered as a control group whereas FM and FF served as treatment group.

The majority (> 77.2%) of the samples from BM, FM and FF was positive for enterococci in high concentrations. Although only 37.7% BF were positive for enterococci, the concentration was also very high in the fly digestive tract: 10^5-10^6 CFU/BF which is similar to results in other studies (Macovei and Zurek, 2006).

The majority of isolates from BM were *E. casseliflavus* followed by *E. faecalis and E. faecium*. This large portion of *E. casseliflavus* is likely due to the association of this species with plants (Muller et al., 2001). Since American bison are grazers on Konza Prairie they likely ingest

E. casseliflavus frequently. There was no difference between *E. cassseliflavus* prevalence between BM and BF isolates (P = 0.99). However, many BF were negative for enterococci, most of the species in positive BF were *E. faecalis* (57.6%) which indicates that many of these flies did not develop in bison manure. Some flies may have developed in garbage and food trash close to the office and human dwellings on the Konza Biological Station or even further away in feces of pastured or confined cattle and other animals.

Diversity of enterococci in FM was very low and that was reported previously from adult dairy cattle (Aarestrup et al., 2001). Moreover, *E. hirae* was the second most prevalent species in calves (Devriese et al., 1992) though reports are available with data on *E. faecalis* and *E. faecium* present in cattle feces (Devriese et al., 1992; Thal et al., 1995). *Enterococcus faecalis, E. faecium, E. hirae* and *E. durans* were most commonly found in farm animal intestines, including cattle (Simjee et al., 2006). In FM, almost all species (91.9%) were *E. hirae* as opposed to FF isolates that showed a broader and more even diversity that included *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus* and *E. hirae.* My house fly data showed species diversity similar to previous studies (Macovei and Zurek, 2006). Overall, enterococcal species distribution showed very interesting results that did not match the study hypothesis. The species diversity in FM strongly suggests that FF did not develop in the feedlot cattle manure. FF either developed in nearby farms or after emergence flew to other animal production sites where they acquired other enterococcal species and returned to the cattle feedlot.

Antibiotic susceptibility test data reflect the antibiotic use in cattle. Ninety one percent of all FM isolates were resistant to tetracycline and 75% were resistant to erythromycin. These two types of drugs are FDA approved (tetracycline, tylosin as well as sulfa drugs and tiamulin) for use in cattle (FDA, 2007) and they are commonly used as growth promoters. Erythromycin and

tetracycline resistance in farm animals has been commonly associated with antibiotic use in animals (Bogaard and Stobberingh, 2000; Johnston and Jaykus, 2004). High tetracycline resistance is also very common in clinical isolates (Jones et al., 1998). In our study, *E. hirae* were the most dominant species and these exhibited very high resistance (94.0%) to tetracycline and erythromycin (77.7%).

In contrast, very low resistance but surprisingly still detectable was observed in BM isolates, especially for tetracycline (3.1%) and erythromycin (19.2%) suggesting that there is an influx of antibiotic resistant strains to Konza Prairie from elsewhere. Relatively high level resistance to ciprofloxacin has been found in BM samples. No sequencing has been done to identify the *ParC* or *GyrA* gene alterations for ciprofloxacin resistance . This finding certainly warrants further investigations.

Interestingly, the difference in resistance profiles between BM and BF isolates was highly significant ($P<0.05$-0.0001) with the exception of streptomycin, ciprofloxacin and ampicillin. This supports the hypothesis that BF did not develop in BM and BF dispersed from the nearby farms or picked up the resistant strains from human residences around Konza Prairie. Furthermore, it is also possible that BF are the vector and source for antibiotic resistant enterococci in BM and represent one of the sources of the influx of antibiotic resistant strains to Konza Prairie.

FF isolates showed significantly higher ($P<0.00001$) phenotypic resistance compared to BF for tetracycline, erythromycin, vancomycin, kanamycin, ciprofloxacin, and chloramphenicol suggesting that BF are a subpopulation of FF. When FF disperse, their gut microbial community may be changing depending on the food sources.

In this study, very low level vancomycin resistance was observed and it was associated mainly with the *E. casseliflavus* in BM, BF, FF samples. *E. casseliflavus* and *E. gallinarum* confer the intrinsic resistance to vancomycin with *vanC* (Kak and Chow, 2002). No vancomycin resistance was found in *E. faecium*. This species poses a great risk with van-resistance and it has been reported to transiently colonize the human gut and transfer the *vanA* gene to human colon microbiota (Sorensen et al., 2001; Lester, 2006). Vancomycin resistance was detected in several *E. faecalis* isolates from BF and this is a subject of further studies (detection of *van* genes) in laboratory.

Regardless of the species diversity, antibiotic resistance showed very similar profiles ($P = 0.1$) in FM and FF isolates except for ampicillin ($P < 0.01$) and kanamycin ($P < 0.0001$). Although it is likely that FF did not develop in cattle manure, they could have acquired the resistance strains from manure from other animal productions in the vicinity of the cattle feedlot, namely poultry and swine farm where tetracycline and tylosin are also used. Additional studies using for example PFGE (pulsed field gel electrophoresis) genotyping will be required to test this hypothesis.

Screening of resistance genes revealed that the most commonly found combination is *tet*M with *ermB* and Tn*916* and Tn*1545/916* family. In FM isolates, identified *tet* genes (M, S, O) and *ermB* were phenotypically expressed in 72.2%-100% isolates. All the *tet* genes and *ermB* that were identified in FF isolates were 100% phenotypically expressed. Isolated *tet* genes are *tetM*, *tetS*, *tetO*, *tetK* from all the samples. Tn*916* and Tn*1545/916* family were frequently detected with *tetM* gene. A very interesting trend was observed between FM and FF samples. The most prominent species, *E. faecalis* in FF and *E. hirae* in FM, showed very similar trend in carrying the resistance genes and transposons. These results indicate that these isolates share the

resistance genes and transposons. No *tetA, tetC, tetQ, tetW* were detected in any FM or FF isolates. Clearly, BM and BF isolates had lower resistance gene profile with transposons than cattle feedlot samples and some of these genes were phenotypically expressed. In BM and BF samples, *tetM* was the most commonly observed resistance genes which exhibited 100% phenotypic expression, other commonly found resistance genes were *tetO, ermB* with Tn*916.* No *tetA, tetC, tetQ* and *tetS* were detected in BM or BF samples. Tn*916* and Tn*1545/916* frequently carries *tetM* (Weaver et al., 2002). This conjugative transposon is likely to mediate tetracycline resistance by *tetM* and Tn*1545* mediates resistance to kanamycin and erythromycin. Having a very broad range, this transposon can transfer even between Gram positive and Gram negative bacteria (Bertram et al., 1991; Poyart et al., 1995). At this point the origin and acquisition of *tetM, ermB* with Tn*916* in BM isolates can not be explained although it is possible that house flies carried the strains with these genes to Konza prairie from areas with high prevalence of antibiotic resistant strains.

Similar to other studies (Hew, 2006, Macovei and Zurek, 2006), the majority of *E. faecalis* (100%) in BM showed proteolytic (gelatinase) activity on 1.5% milk agar plates and 79.2% of *E. faecalis* from BF also exhibited gelatinase activity. No significant differences were observed (P=0.21) between BM and BF isolates regarding gelatinase phenotype. No proteolytic activity was observed in FM but in FF samples, the majority of *E. faecalis* (72.5%) showed gelatinase activity. This indicates that gelatinase is widely spread among environmental enterococci and these may represent a reservoir for clinically relevant enterococcal strains.

Clumping assay for detection of the phenotypic aggregation substance was also conducted. Only *E. faecalis* from house flies from both sites showed clumping referring to the fact that flies are carrying phenotypically virulent strains as compared with the animal fecal

samples. *E. faecalis* from BF (5.6%) and FF (5.5%) isolates exhibited cell aggregation. *E. faecium* (10%) isolates from BM showed hemolytic activity. In BF isolates, 39.6% of *E. faecalis* were hemolytic on human blood. *E. casseliflavus* (14.3%) from FM was positive for hemolysis on human blood. In FF, almost a half of *E. faecalis* (45.0%) were hemolytic on human blood. Phenotypic data of virulence factors did not show a very different trend. Virulence factors were distributed among all samples from BM, BF, FF and FM. Flies from both sites tended to carry more hemolytic strains than manure isolates ($P<0.0001$). FF isolates were positive for virulent phenotypes more frequently than BF isolates ($P<0.01$) especially for hemolytic and gelatinase activity.

Correspondingly, the most abundant virulence gene was *gel*E which was found in *E. faecalis* from all samples. *E. faecalis* (73.6% from BM) and BF (75.5% from BF) carried *gel*E which was commonly expressed (100% in BM and 87% in BF). In FF, *gel*E was found in all species but the majority of *gel*E was detected in *E. faecalis* (82.5%) and it was phenotypically expressed (72.7%). Among FF isolates, the highest combination of virulence factors was found in *E. faecalis* (2.5%) with *gel*E, *asa*1 and *esp*. In BF, one *E. faecalis* (0.9%) also carried *esp* with *gel*E. *E. faecalis* (5.6%) was found to have two virulence factors *gel*E and *asa*1 (33.3% phenotypically expressed). Two isolates in *E. hirae* and one in *E. casseliflavus* from FM had the *cyl*A gene. Cytolysin toxin was used to be described only in *E. faecalis* (Hancock and Gilmore, 2000) but other species such as *E. hirae* and *E. casseliflavus* also have been investigated and were found with complete hemolysis activity and c*yl* genes (Semedo et al., 2003). *E. faecalis* with *gelE* and *esp* is associated with clinical isolates and biofilm formation (Toledo-Arana et al., 2001; Shankar et al., 2001; Hancock and Perego, 2004).

The data of genotypic screening of enterococcal virulence factors supports our phenotypic results and confirms that some environmental enterococcal strains accumulate virulence factors and may become clinically important. The functional significance of these factors in the non-clinical environment remains to be investigated but it may involve biofilm formation (*gelE, esp*), nutritional advantages (*gelE*), horizontal gene transfer of resistance genes (*asa1*) during conjugation, and other potentially fitness enhancing strategies.

Persistence of antibiotic resistance in farm animals makes this scenario more serious and complicated. After the use for several years, Norway, Germany, Denmark and all other European Union countries banned glycopeptide avoparcin and all other antibiotics for the use as growth promoters in 1997. This was due to reports of high prevalence of *van-A* type glycopeptide – resistant enterococci in farm animals and the connection between resistance development and avoparcin use and furthermore vancomycin resistance in community isolates (Sørum et al., 2006).

In this study, our results clearly indicate the antibiotic selective pressure has an influence on the prevalence of resistant bacteria. Also, low level resistance and associated resistance and virulence genes in bison manure was not expected. From this data, the origin (developmental site) of house flies collected from the cattle feedlot and Konza prairie and the associated enterococci can not be determined. Nevertheless, enterococci from house flies were found to be more associated with resistance and virulence genes of clinically important strains. House fly dispersal ability makes this insect likely to transfer and disseminate antibiotic resistance and virulence genes from the farm environment to urban settings and therefore an important vector from the public health perspective.

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Table 1. Prevalence of enterococci in house flies and manure from cattle feedlot and Konza Prairie Nature Preserve

Source	No. samples analyzed/ positive (%)	CFU per HF or g feces Mean ± SE	No. isolates	No. identified isolates (%)	Identified isolates (%)				
					E. faecalis	*E. faecium*	*E. casseliflavus*	*E. gallinarum*	*E. hirae*
Konza Manure (BM)	23/23 (100)	$5.1 \pm 3.8 \times 10^6$	167	125 (74.8)	19 (15.2)^{aA}	10 (8.0)^{A}	73 (58.4)^{aA}	0	15 (12.0)^{aA}
House flies (BF)	114/43 (37.7)	$1.4 \pm 0.5 \times 10^5$	141	92 (65.2)	53 (57.6)^{bA}	0	39 (42.3)^{aA}	0	1 (0.9)^{aA}
Feedlot Manure (FM)	110/85 (77.2)	$4.2 \pm 0.1 \times 10^5$	189	112 (59.2)	1 (0.8)^{aB}	1 (0.8)^{aB}	7 (6.3)^{aB}	0	103 (91.9)^{aB}
House flies (FF)	124/121 (96.8)	$4.8 \pm 0.9 \times 10^6$	223	112 (50.2)	40 (35.7)^{bA}	24 (21.4)^{b}	20 (17.9)^{bB}	22 (19.6)	8 (7.1)^{bB}

Values within the same source and different type of samples followed by the same lowercase letter are not significantly different and values of the two different sources and same type of samples followed by the same capital letter are not significantly different ($p > 0.05$; PROC T-Test: SAS Institute 2003).

Table 2. Correlation of genotype and phenotype characteristics of cattle manure and houseflies from feedlot

Combination of determinants	Feedlot manure		House flies	
	Total no. (% positive)	% correlation with phenotype	Total no. (% positive)	% correlation with phenotype
E. hirae	**n=103**		**n=8**	
tetM plus				
tetO + Tn916	1 (0.9)	100/ NA	0	
ermB + Tn 916	18 (17.5)	94.4/ 72.2/ NA	0	
ermB	2 (1.9)	100	0	
Tn916/Tn1545				
family	1 (0.9)	NA	0	
Tn916	12 (11.7)	NA	3 (37.5)	33.3
tetO plus				
ermB + Tn916	11 (10.7)	100/ 100/ NA	0	
ermB +				
Tn916/Tn1545	4 (3.9)	100/ 100/ NA	0	
ermB	18 (17.5)	100/ 88.9	0	
tetS + erm B	1 (0.9)	100/ 100	0	
tetM alone	6 (5.8)	100	2 (25)	50
tetS alone	1 (0.9)	100	0	
tetO alone	6 (5.8)	100	0	
ermB alone	10 (9.7)	70	2 (25)	0
Tn916 alone	1 (0.9)	NA	0	
Tn916/Tn1545 alone	1 (0.9)	NA	0	
E. faecalis	**n=1**		**n=40**	
tetM plus				
tetO + ermB +				
Tn916/ Tn1545	0		1 (2.5)	100/ 100/ NA
ermB + Tn916/				
Tn1545 family	0		2 (5.0)	100/ 100/ NA
ermB + Tn916	0		1 (2.5)	100/ 100/ NA
ermB	0		1 (2.5)	100/ 100/ NA
Tn916/Tn1545				
family	0		9 (22.5)	100/ NA
tetO + ermB	1 (100)	100	1 (2.5)	100/100
ermB + Tn 916	0		1 (2.5)	100/ NA
ermB + Tn916/Tn1545				
family	0		1 (2.5)	100/ NA
tetM alone	0		2 (5.0)	100
ermB alone	0		3 (7.5)	100
Tn916 alone	0		2 (5.0)	NA

E. faecium	**n=1**		**n=24**	
tetM plus				
Tn916/Tn1545	0		1 (4.2)	0/ NA
Tn916	0		1 (4.2)	100/ NA
tetK	1 (100)	100		
tetM alone	0		8 (33.3)	87.5
tetS alone	0		2 (8.3)	50%
E. casseliflavus	**n=7**		**n=20**	
tetM plus				
ermB +				
Tn916/Tn1545	0		1 (5.0)	100/ 100/ NA
ermB	0		1 (5.0)	100/ NA
Tn916	1 (14.3)	100/ NA	1 (5.0)	NA
tetO plus				
tetS + ermB	1 (14.2)	100/0	0	
ermB	1 (14.2)	0/ 0	0	
tetS + ermB	1 (14.2)	100/ 100	0	
tetM alone	0		1 (5.0)	100
ermB alone	0		3 (15.0)	100
tetO alone	3 (42.8)	33.3	0	
E. gallinarum	**n=0**		**n=22**	
tetM plus	NA			
Tn916/Tn1545			3 (13.6)	100
tetM alone	NA		1 (4.5)	100
tetS alone	NA		1 (4.5)	100
tetO alone	NA		2 (9.1)	50
Tn916/Tn1545 alone	NA		1 (4.5)	NA

Table 3. Correlation of genotype and phenotype characteristics of enterococci from bison manure and house flies from Konza Prairie

Combination of determinants	Bison manure		Konza house flies	
	Total no. (% positives)	% correlation with phenotype	Total no. (% positives)	% correlation with phenotype
E. hirae	**n=15**		**n=1**	
tetM plus				
ermB + Tn916	2 (13.3)	0/ 100/ NA	0	
Tn916	1 (6.7)	100/ NA	0	
tetO alone	1 (6.7)	100	0	
Tn916 alone	1 (6.7)	NA	1 (100)	NA
E. faecalis	**n=19**		**n=53**	
tetM plus				
ermB + Tn916	0		1 (1.9)	100/ 100/ NA
tet K + Tn916	1 (5.2)	0	0	
tetK + Tn1545/ Tn916	1 (5.2)	0/ NA	0	
tetM alone	2 (10.5)	0	0	
tetK alone	6 (31.5)	0	0	
Tn1545/Tn916 alone	1 (5.2)	NA	0	
Tn916 alone	1 (5.3)	0	1 (1.9)	NA
E. faecium	**n=10**		**n=0**	
tetM + tetK	1 (10.0)	0/0	NA	
te M alone	3 (30.0)	0	NA	
tetK alone	3 (30.0)	0	NA	
Tn1545/Tn916	0		NA	
E. casseliflavus	**n=73**		**n=39**	
tetM plus				
Tn916	1 (1.4)	100/ NA	0	
tetO + erm B	1 (1.4)	0/ 0	0	
tetM alone	14 (19.2)	0	1 (2.6)	100
tetO alone	1 (1.4)	0	6 (15.4)	0
tetS alone	0		3 (7.7)	66.7
Tn1545/Tn916	1 (1.4)	NA	0	

Table 4. Correlation of genotype and phenotype characteristics of virulence factors of enterococci from feedlot cattle feces and house flies.

Virulence Genes	Number (%) of isolates					% Correlation with phenotype				
	E. hirae	*E. faecalis*	*E. faecium*	*E. casseliflavus*	*E. gallinarum*	*E. hirae*	*E. faecalis*	*E. faecium*	*E. casseliflavus*	*E. gallinarum*
	FM/ FF*	FM/ FF	FM/ FF	FM/ FF	FM/ FF	FM/ FF	FM/ FF	FM/ FF	FM /FF	FM/ FF
gelE	0/ 0	1 (100) / 33(82.5)	0 / 8 (33.3)	0/ 3 (15)	0/ 90 (40.9)	NA	100/ 72.7	NA/ 25	NA/ 33.3	NA/ 33.3
asa1	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	NA	NA#	NA	NA	NA
esp	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	NA	NA	NA	NA	NA
cylA	2 1.9/ 0	0/ 0	0/ 0	1 (14.3)/ 0	0/ 0	0/ NA	NA	NA	0/ NA	NA
gelE + *asa1*+ *esp*	0/ 0	0 / 1 (2.5)	0 / 1 (4.7)	0/ 1 (5)	0/ 0	NA	NA/ (0/0/0)	NA/ (0/0/0)	NA/ (100/0/0)	NA
gelE + *asa1*	0/ 0	0 (0.0) / 1 (2.5)	0/ 0	0/ 0	0/ 0	NA	NA/ (0/0)	NA	NA	NA
gelE + *esp*	0/ 0	0/ 0	0/ 0	0/ 3(15)	0/ 2(9.1)	NA	NA	NA	NA/ (33.3/ 0)	NA/ (100/0)

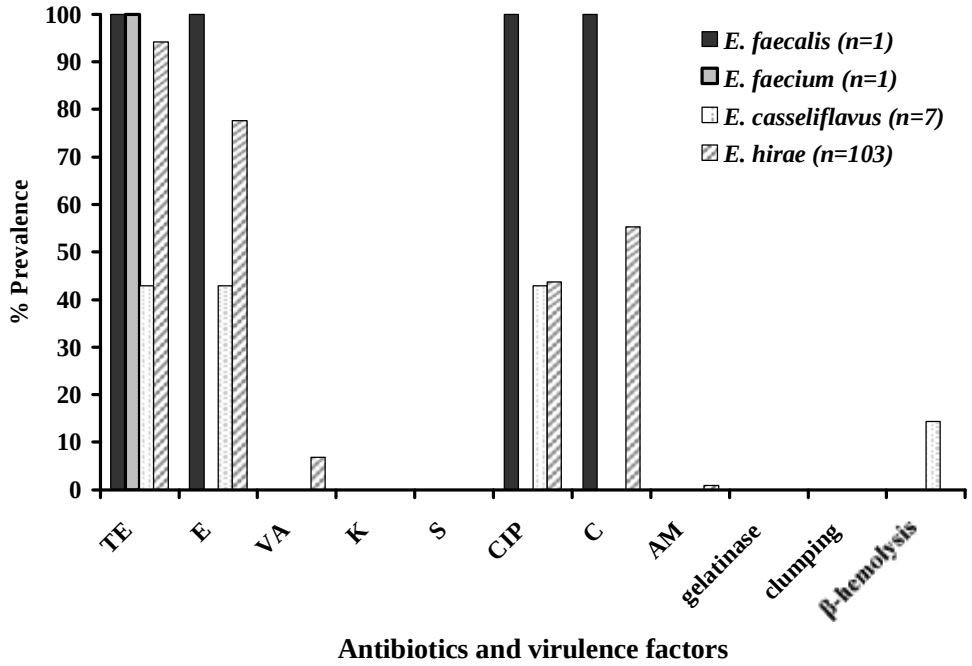
*FM= cattle manure, FF= house flies from feedlot # NA=Not applicable

Table 5. Correlation of genotype and phenotype characteristics of virulence factors of enterococci from bison feces and houseflies from Konza Prairie

Virulence genes	Number (%) of isolates				% Correlation with phenotype			
	E. hirae	*E. faecalis*	*E. faecium*	*E. casseliflavus*	*E. hirae*	*E. faecalis*	*E. faecium*	*E. casseliflavus*
	FM/ FF*	FM/ FF	FM/ FF	FM/ FF	FM/ FF	FM/ FF	FM /FF	FM/ FF
gelE	0/ 0	14 (73.6)/ 40 (75.5)	1 (10)/ 0	4 (15.2)/ 0	NA#	100/ 87.5	100/ NA	100/ NA
Asa1	0/ 0	0/ 0	0/ 0	0/ 0	NA	NA	NA	NA
Esp	0/ 0	0/ 0	0/ 0	0/ 0	NA	NA	NA	NA
Cyl	0/ 0	0/ 0	0/ 0	0/ 0	NA	NA	NA	NA
gelE + *asa1*	0/ 0	0/ 3 (5.6)	0/ 0	0/ 0	NA	NA/ (100/33.3)	NA	NA
gelE + *esp*	0/ 0	0/ 1 (0.9)	0/ 0	0/ 0	NA	NA/ 0	NA	NA

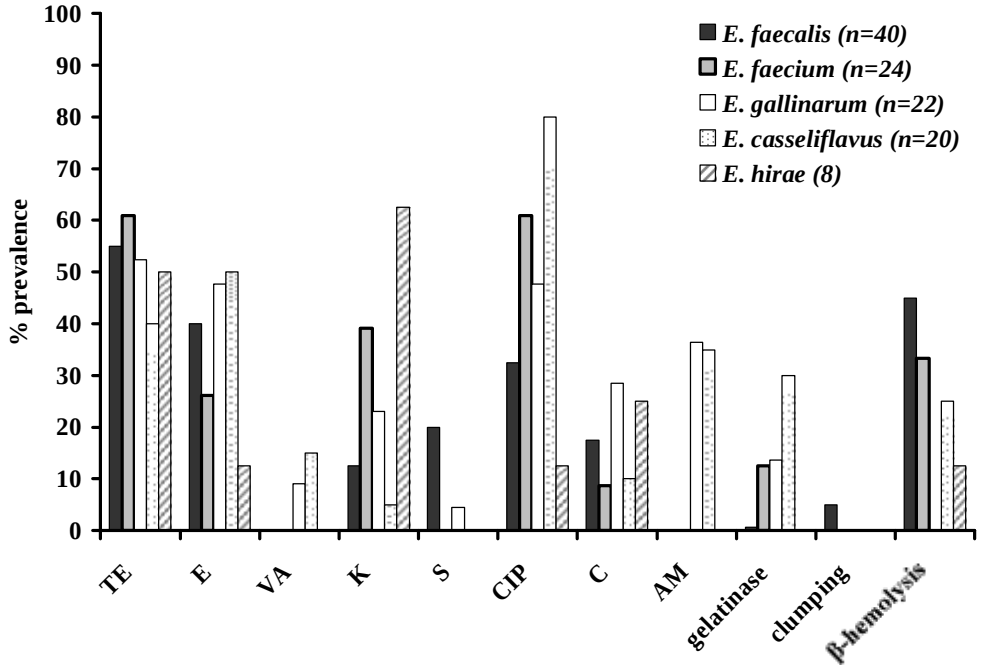
*BM = Bison manure, BF = house flies from Konza; # NA=Not applicable

Fig. 1. Antibiotic resistance and virulence phenotypic profiles of enterococci from feedlot cattle manure



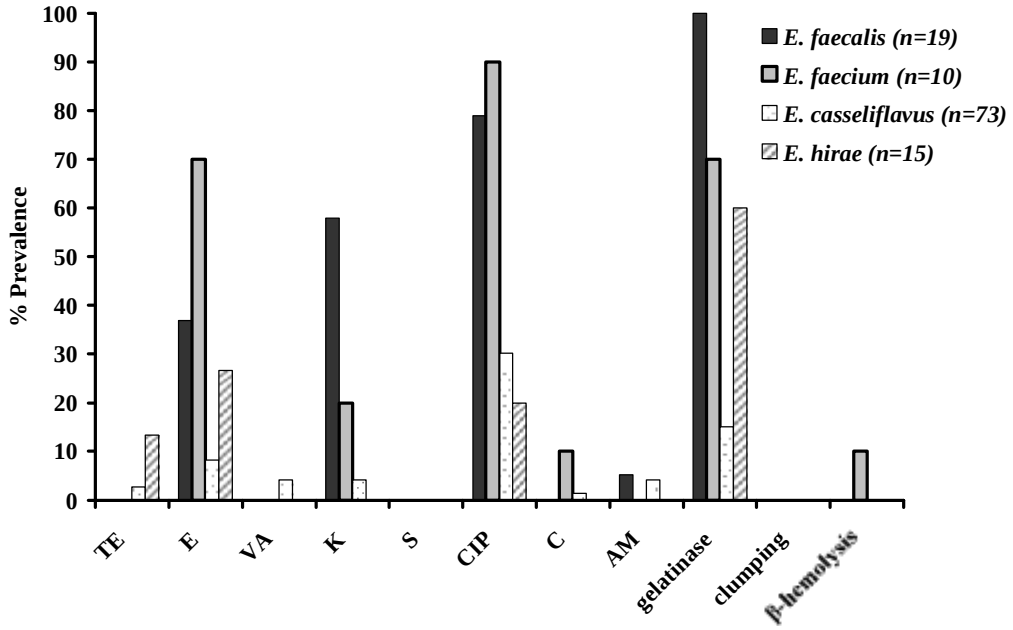
* TE=Tetracycline, E=Erythromycin, VA=Vancomycin, K=Kanamycin, S=Streptomycin, CIP= Ciprofloxacin, C= Chloramphenicol, AM= Ampicilin

Fig. 2. Antibiotic resistance and virulence phenotypic profiles of enterococci from houseflies from the feedlot



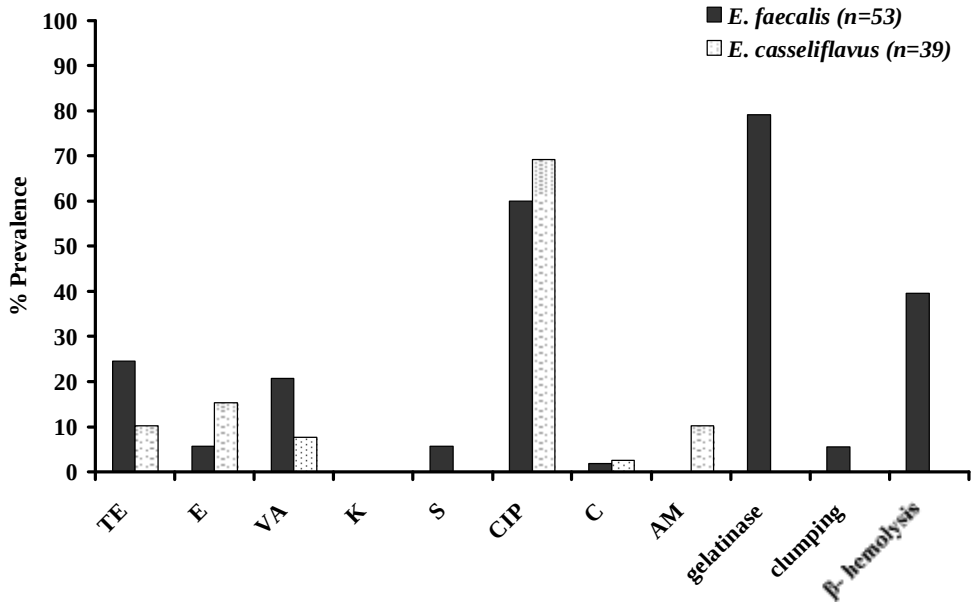
* TE=Tetracycline, E=Erythromycin, VA=Vancomycin, K=Kanamycin, S=Streptomycin, CIP= Ciprofloxacin, C= Chloramphenicol, AM= Ampicilin

Fig. 3. Antibiotic resistance and virulence phenotypic profiles of enterococci from bison manure



* TE=Tetracycline, E=Erythromycin, VA=Vancomycin, K=Kanamycin, S=Streptomycin, CIP= Ciprofloxacin, C= Chloramphenicol, AM= Ampicilin

Fig. 4. Antibiotic resistance and virulence phenotypic profiles of enterococci from house flies from Konza Prairie Preserve



* TE=Tetracycline, E=Erythromycin, VA=Vancomycin, K=Kanamycin, S=Streptomycin, CIP= Ciprofloxacin, C= Chloramphenicol, AM= Ampicilin

CHAPTER 3

Assessment of the potential for horizontal gene transfer among enterococci in the house fly (*Musca domestica* L.) digestive tract

ABSTRACT

In the context of heavy antibiotic use and rapid emergence of resistant bacteria, horizontal transfer of resistance genes is a very important area of research. As a part of an *Enterococcus*-house fly symbiosis study, the house fly digestive tract was evaluated for the potential of horizontal transfer of the antibiotic resistance gene *tetM* on the plasmid pCF10 among *Enterococcus faecalis* strains. The house fly digestive tract is of a particular interest due to house fly larval habitat (decomposing organic material with large and diverse microbial communities), unrestricted movement of adult flies, modes of feeding (regurgitation), and attraction to human food and drinks. Moreover, no reports are available for horizontal transfer of resistance genes among enterococci or any other Gram- positive bacteria in the house fly digestive tract.

In this experiment, house flies were exposed to the donor (*E. faecalis* OGIRF:pCF10) (D) for 12 hrs and the recipient (*E. faecalis* OGISSP) (R) inocula (DR group) for 1 hr and another group received the recipient first and the donor second (RD group). The flies were screened daily to determine donor, recipient and transconjugants (transfer of pCF10 plasmid with *tetM*) for 5- 6 days. Both groups of flies (DR, RD) (n= 90/group) showed that transfer that occured 24 hrs after exposure with the rate up to 10^{-1} CFU transconjugant per donor (T/D)**.** There was no significant difference (P>0.05) in transfer rate between these two groups of flies. The corresponding water (drinking water for flies) samples were also tested and positive for transconjugants but their consistent appearance after the conjugation in flies strongly indicated that the transfer took place within flies first. In the next set of experiments, the conditions were the same as previously but the flies (n= 75/group) were dissected to separate the labellum and rest of the fly body was surface sterilized to determine the site of the conjugation within the flies. Transconjugants were

isolated from the flies after 24 hrs of bacterial exposure. Interestingly, transconjugants were found in labella of both groups (DR and RD) with a transfer rate up to 10^1 T/D. In this case, the transconjugant transfer frequency was higher ($P<0.01$) in DR group (34%) of flies than RD group (3%). In surface-sterilized flies, transconjugant transfer rate (T/D) ranged from 10^{-5} to 10^1 CFU in the DR group and 10^{-3} to 10^{-1} CFU in the RD group. Transfer of transconjugants was significantly higher ($p<0.01$) in DR group (52%) of flies than RD group (19%). Water samples were again inconsistently positive for transconjugants from day 2. The high concentration of transconjugants in the surface-sterilized flies clearly indicated that the conjugation took place in the fly digestive tract and flies likely contaminated the water during drinking. It is difficult to make any conclusions about the higher transfer rate in DR group of flies than in the RD group. Overall, the transfer rate was very high as much as 10^1 T/D which may be due to growth of the transconjugant population in the fly gut and/or drinking the contaminated water. Non-surface sterilized and surface sterilized flies were positive for transconjugants for the following 4-5 days of inoculum exposure. This has great implications for public health. All these data suggest that the house fly digestive tract provides the conditions for horizontal transfer of resistance genes among enterococci which underscores the importance of this insect as a potential vector of antibiotic resistant bacterial strains.

INTRODUCTION

Acquisition of antimicrobial resistance occurs very frequently in nature and resistance genes can spread very fast in bacterial populations by horizontal gene transfer (Hall, 2004). Many studies have been conducted to determine the origin and fate of pathogens (acquisition of virulence genes) during evolution as well as for treatment and control of the infections (acquisition of antibiotic resistance genes) (Rice, 2000; Elsas and Bailey, 2002; Choi, 2007). There are different mechanisms by which bacteria can transfer DNA with resistance and virulence genes (Murray, 1998; Rice, 2000) and this includes mainly conjugation, transduction, and transformation. Resistance gene transfer in gram-positive bacteria occurs mainly by conjugation through plasmids and transposons (Rice, 2000; Elsas and Bailey, 2002). This can, for example, involve pheromone responsive plasmids, non-responsive conjugative plasmids, non-conjugative mobilizable plasmids, and conjugative transposons (Rice, 2000; Elsas and Bailey, 2002).

Enterococci are intrinsically resistant to several antimicrobials (e.g. low concentration aminoglycosides, quinolones, and third generation of cephalosporins) (Ogier and Serror, 2007). In addition, enterococci frequently acquire antibiotic resistance (glycopeptides, tetracyclines, macrolides) and virulence determinants via conjugation through mobile genetic elements such as plasmids and transposons. There are no well-documented reports of gene transfer by natural transformation or transduction in enterococci (Jacobs and Hobbs, 1974; Clewell and Dunny, 2002). Plasmids of enterococci may carry and encode the transfer of antibiotic resistance determinants, hemolysins, bacteriocins and they can also induce the movement of chromosomal determinants (Franke et al., 1978; Murray, 1998; Clewell and Dunny, 2002). In enterococci, three different self-replicating plasmids include rolling circle replicating plasmids (RCR), Inc18

plasmids, and the pheromone responsive plasmids (Weaver et al. 2002). The RCR and Inc18 plasmids can replicate in a broad host range but pheromone responsive plasmids are conserved to enterococci (Murray, 1998; Weaver et al., 2002). *Enterococcus faecalis* exhibits pheromone responsive conjugation by which it can transfer resistance and virulence genes by plasmids at high frequency within the same species (Murray 1998, Buttaro et al., 2000). Enterococci also can transfer the broad host range plasmids by a non-pheromone responsive conjugation system by which the plasmid can be transferred to other species as well as other gram-positive genera such as streptococci and staphylococci. However, in such conjugation system, the transfer rate is much lower than that of pheromone responsive mechanisms (Clewell, 1981). The transfer of *vanA* Tn*1546* by *E. faecalis* plasmid pAM830 to *Staphylococcus aureus* is an example of such transfer (Sung and Lindsay, 2007). Another type of conjugation in enterococci involves the conjugative transposons. Conjugative transposons are not self-replicable but they have the ability to integrate into the host chromosome or plasmid hence posses more risk to transfer the resistance genes to Gram-positive as well as Gram-negative bacteria (Roberts, 1990a, Roberts, 1990b).

In this experiment, the principles of pheromone responsive plasmid conjugation system were adopted.

Pheromone-responsive plasmid conjugation: Pheromone responsive plasmid conjugation is so far the best known conjugation system in *E. faecalis* which involves oligopeptide pheromones and pheromone responsive plasmid (Clewell and Keith, 1989). The plasmid pCF10 of *E. faecalis* OG1RF is one of the most studied pheromone inducible plasmids and encodes the tetracycline resistance *tetM* (Tomich et al., 1979; Dunny et al., 1981). In this

conjugation system, *E. faecalis* donor cells are induced by specific signaling peptides/pheromones and transfer the conjugative plasmid to *E. faecalis* recipient cells. This process is very specific and a specific single plasmid or closely related family of plasmids can only be transferred in response to its specific pheromone peptides. The plasmid pCF10 transfers by specific induction of the pheromone cCF10 (LVTLVFV) produced by the recipient cells (Buttaro et al., 2000). *Enterococcus faecalis* donor cell exhibits the dual genetic capacity of chromosomal production of cCF10 and plasmid encoded response. In pheromone responsive conjugation, the recipient cells of *E. faecalis* secrete different specific pheromone peptides that interact with specific plasmids (Wirth, 1994). When the potential donor cell interacts with the pheromone (Leonard et al., 1996), the pheromone responsive plasmid induces the *prg* transcriptions. The cCF10 pheromone induces *prg*B gene expression in donor cells which results in synthesis of a sticky substance called the aggregation substance on the cell surface (Olmsted et al., 1991; Chung and Dunny, 1992; Bensing and Dunny, 1997). When the donor cells of *E. faecalis* get into contact with the *E. faecalis* recipient cells, the aggregation substance attaches to the binding substances on the surface of cells. This binding causes clumping of cells in which the donor cells transfer the pheromone responsive plasmid to the recipient cell (Dunny et al., 1978; Olmsted et al., 1991; Bensing and Dunny, 1997; Murray, 1998; Hirt et al., 2005). When the recipient cells receive the plasmid, plasmid transfer stops though aggregation substances are on the cell surface for long time in pCF10 (Hirt et al., 2005).

Several studies of horizontal gene transfer of bacteria have been conducted in laboratory and in the field. The ecological studies have been done to highlight the role of environmental factors such as temperature, moisture, nutrients, competing populations and transfer rates. Emergence of VRE (Vancomycin resistant enterococci) nosocomial infections in the USA and

spreading *vanA* to other bacteria such as *Staphylococcus aureus* are serious threats to human health. Being recognized as a growing concern of multi-drug resistance microbes, horizontal transfer of antibiotic resistance genes in enterococci has been investigated in intestinal microbiota (Sung and Lindsay, 2007) of different hosts. These studies involved the transfer of *vanA* of *E. faecium* from porcine to human microbiota (Moubareck et al., 2003), transfer of *vanA* and *vanB* in mice digestive tract (Dahl et al., 2007), transfer of the *vanA* resistance gene among *E. faecium* in the intestine of human volunteers (Lester et al., 2006), and transfer of the *tetM* and *ermB* resistance genes from *Lactobacillus plantarum* to *Enterococcus faecalis* JH2-2 in gastrointestinal tract of gnotobiotic rats (Jacobsen et al., 2007).

In this project, the house fly gut was evaluated as potential site for horizontal gene transfer among enterococci. The horizontal gene transfer in the house fly digestive tract is of particular interest due to several factors. Several studies have suggested that house flies pose a high risk to human health by carrying resistant and potentially virulent strains of enterococci in the urban environments (Graczyk et al., 2001; Macovei and Zurek, 2006). House flies are the most important non-biting insect pest of public health in their role as mechanical vectors of potential pathogens. Development of house fly larvae occurs in various decaying organic substrates such as manure and garbage with rich microbial communities (Schmidtmann and Martin, 1992; Zurek et al., 2000). Therefore, house flies are likely to carry a diverse microbial population in their digestive tracts. Adult house flies have been reported to carry and transmit *Yersinia pseudotuberculosis, Helicobacter pylori, Campylobacter jejuni, Escherichia coli* O157:H7, *Salmonella* spp., *Aeromonas caviae, Enterococcus spp.* (Greenberg, 1971; Shane et al., 1985; Moriya, 1999; Sasaki et al., 2000; Graczyk, et al., 2001; Zurek et al., 2001; Alam and Zurek, 2004). The habitat, feeding habit by regurgitating, high dispersal ability, close contact

with humans, free movement from animal farms to human residences make them more important as vectors of bacteria originating from animal manure and other decaying organic substrates.

Several studies have suggested that the digestive tract of some insects serves as a site for horizontal gene transfer (Jarrett et al., 1990; Hoffmann et al., 1998; Watanabe et al., 1998; Hinnebusch et al., 2002; Dillon and Dillon, 2004; Petridis et al., 2006). The bacterial community in the insect gut can withstand antibiotic selective pressure and adapt to the microenvironment by continuous transfer of plasmids and other mobile genetic elements. *Escherichia coli* demonstrated transfer of the conjugative plasmid pRP4-luc to several proteobacterial species in the gut of *Folsomia candida* (Collembola) (Hoffmann et al., 1998). *Bacillus thuringiensis* plasmid transfer was successful between strains in the gut of *Galleria mellonella* and *Spodoptera littoralis* (Jarrett et al., 1990). The plasmid (conjugative plasmid pBPW1::Tn*7*) was also transferred between *Erwinia herbicola* strains in the gut of silkworm (*Bombyx mori*) larvae (Watanabe et al., 1998). Horizontal transfer of the antibiotic-resistance plasmid of *E. coli* to *Yersinia pestis* was demonstrated in the cat flea (*Ctenocephalides felis*), midgut (Hinnebusch et al., 2002). House flies have long been known as a mechanical vector for various bacteria and other microorganisms. One recent study reported the horizontal transfer of antibiotic resistance genes and virulence genes among *E. coli* strains in the house fly gut (Petridis et al., 2006).

So far, no reports are available for horizontal transfer of antibiotic resistance genes or any other genes among Gram-positive bacteria in the house fly gut. In this study, horizontal transfer of the antibiotic resistance gene among *Enterococcus faecalis* strains in the house fly gut was investigated.

OBJECTIVE

To determine the potential for horizontal gene transfer among *E. faecalis* strains in the house fly digestive tract

HYPOTHESIS

Horizontal transfer of antibiotic resistance genes takes place in the house fly digestive tract

MATERIALS AND METHODS

Pheromone-responsive plasmid conjugation

The pheromone responsive plasmid conjugation system of enterococci was used to conduct the horizontal transfer of the plasmid pCF10 from *E. faecalis* OGIRF:pCF10 to *E. faecalis* OGISSp.

Experimental design

Two different types of bioassays were conducted. In the first experiment, house flies were analyzed every day for 5 days for donor, recipient and transconjugants without surface sterilization and dissection. Five flies were processed every day from two different groups of flies: 1) Exposure to donor first, recipient second (DR group) and 2) Exposure to recipient cells first and donor second (RD group). Three replications (n = 75) were conducted for each group.

In the second experiment, the fly labellum was separated and analyzed separately from the rest of the body. The rest of the fly body was surface sterilized before further processing. Labellum and fly body then were processed to isolate donor, recipient, and transconjugant cells.

Every day 5 flies were processed and three replications (n = 75) were done for both groups, DR and RD.

***Enterococcus faecalis* strains**

E. faecalis OGIRF:pCF10 was used as a donor strain. This strain has the rifampicin resistance gene on the chromosome and the tetracycline resistance gene *tetM* on the plasmid pCFI0. *Enterococcus faecalis* OGISSp was used as a recipient strain with streptomycin resistance gene on the chromosome (OGISSp). All strains were cultured on THB with antibiotics to confirm the viability of resistance genes before experiments. Fresh bacterial culture was used for each experiment.

Selective media

m-*Enterococcus* agar plates with rifampicin (50 mg/L) and tetracycline (4.0 mg/L) were used to isolate donor cells, *E. faecalis* OGIRF:pCF10. m-*Enterococcus* agar plates with streptomycin (2.0 g/L) antibiotics was used for recipient cells, *E. faecalis* OGISSp. m-*Enterococcus* agar plates with streptomycin (2.0 g/L) and tetracycline (4.0 mg/L) for transconjugants. All agar plates were made following the agar dilution method.

***In vivo* plasmid transfer experiments**

Fresh house fly pupae were obtained from the laboratory house fly colony (origin: Beltsville, MD) reared in the Department of Entomology, Kansas State University. All pupae were surface sterilized following a standard procedure (Zurek et al., 2000). All pupae were separated into two groups and transferred to two sterile Petri dishes and kept at room temperature (~25^{o}C) in an incubator for adult emergence.

Two fresh cultures of donor (OGIRF:pCF10) and recipient (OGISSp) cells were made by inoculating from fresh pure culture plates supplemented with antibiotics. Donor and recipient cultures were grown in 5 ml TSB media at 37^{o}C for 18 hrs. After overnight incubation, all cultures were transferred separately into 2.5 ml Eppendorf tubes and centrifuged for 1 minute at 10,000 rpm. The supernatants were discarded and the pellets were re-suspended in sterilized de-ionized water. The donor and recipient culture turbidities were measured by spectrophotometer and adjusted with sterilized distilled de-ionized water to obtain the same concentrations for both cultures (10^{7} CFU/ml).

As soon as the house flies began to emerge, one group received the donor inoculum (3 ml) plus dry milk and sugar cube *ad libitum* for 12 hrs. At the same time, another group was offered the recipient inoculum (3 ml) for 12 hrs with dry milk and sugar *ad libitum*. Another set of donor and recipient inoculum was prepared in 5.0 ml TSB medium and incubated at 37^{o}C for 18 hrs for the next day.

The donor and recipient inoculums with same concentration were prepared by centrifugation and re-suspension in sterilized water following the same procedures as described above. After the 12 hrs of incubation, house flies that received donor inoculum were offered the recipient inoculum for 1 hr (DR group). In a similar way, after 12 hrs, the fly group that received recipient inoculum was switched to donor inoculum for 1 hr (RD group). After the 1 hr incubation, flies in both group (DR and RD group) were aseptically transferred to deep sterile Petri dishes and separated to 7 house flies/dish with sterile tap water and new foods (sugar and dry milk *ad libitum*). At this time, five flies from each group (DR and RD) were processed immediately to isolate donor, recipient, and transconjugants (as described below).

Two different types of experiments were conducted as described above. Every day five flies, water samples and labella (in one type experiment) from each group were processed for 4 - 5 days. Each fly was homogenized in 1.0 ml phosphate buffer saline (PBS), two 10-fold serial dilutions were made and 34 µL were drop plated on media to isolate donor, recipient and transconjugants. Each day, 1 ml water sample was processed from each group with two 10-fold serial dilutions into PBS and drop plated on three different media to compare the concentration of donor, recipient, and transconjugant cells. Each labellum was homogenized in 200 µl of PBS; two 10-fold serial dilutions were made and processed on three selective media.

Assessment of transconjugants, donor and recipient

Growth of transconjugants was observed daily for both groups for the following 4 -5 days. All bacterial colonies were counted and calculated to determine the concentration of donor, recipient and transconjugant cells.

Data analysis

Spotted graphs were created by InStat 3 software (GraphPad Software, Inc., San Diego, CA). Each graph shows the concentration of donor, recipient and transconjugants for individual fly, water samples, and labella from all fly groups. The normal distribution test was conducted by Chi-square test for discrete variables using WinSTAT Statistics for Excel.

RESULTS

In the non-surface sterilized flies, both the donor and recipient established in a similar concentration and stayed relatively constant throughout the experiment (~10^4 – 10^6 CFU per fly) (Figs. 1, 3). In the DR group, as expected, all flies (N = 15) were negative for transconjugants on Day 0 immediately after the exposure to donor and recipient cells (Fig. 1, Table 1). The first transconjugants were isolated from house flies 24 hr. (Day 1) after the inoculum exposure. On day 1, four of 15 flies were positive for transconjugants (6.0×10^1 - 2.1×10^4 CFU/fly). The range of transfer rate of transconjugants per donor (T/D) was from 1.4×10^{-3} to 8.0×10^{-3} CFU/fly (Figure 1, Table 1). Transconjugants were isolated consistently for the next four days from the flies (Figure 1). The range of transfer frequency of transconjugants per donor (T/D) was 9.6×10^{-3} to 0.3×10^1 CFU/ fly on day 2; 9.3×10^{-4} to 1.9×10^{-2} CFU/ fly on day 3, 7.6×10^{-3} to 1×10^{-1} CFU/ fly on day 4 and 8.6×10^{-3} to 1.5×10^{-1} CFU/ fly on day 5 (Table 1). Overall, 52% of the flies were positive for transconjugants when they were not surface sterilized and received donor inoculum first, recipient second. Also, the corresponding water samples (n=3/day) that were fed to the flies were processed (day 1 to day 5) to detect the donor, recipient and transconjugant levels. Though the water was positive for donor and recipient strains, transconjugant appearance was inconsistent. No transconjugants were present in water on days 1, 2, and 5 (Figure 2). Transconjugants were isolated from water for the first time on day 3 (3×10^1 CFU/ml) and again on day 4 (1.8×10^4 CFU/ml) (Figure 2).

Among the group of non- surface sterilized flies that were fed recipient first donor second (RD group), the concentration of donor and recipient cells was similar to that of DR group throughout the bioassay. No transconjugants were isolated from flies on day 0. Transconjugants were detected for the first time in flies on day 1, 24 hr after the recipient and donor exposure

(Figure 3, Table 3). The transconjugant concentration in flies ranged from $3x10^{1}$ to $6.9x10^{3}$ CFU/fly (Figure 3). Seven out of 15 house flies were positive for the transconjugants on day 1. The transfer rate (T/D) varied from $1.1x10^{-3}$ to $0.1x10^{1}$ CFU/fly. Transconjugants were isolated from flies the following four days with the T/D range ($2.9x10^{-4}$ to $1.2x10^{-1}$ CFU/fly on day 2; $3.2x10^{-3}$ to $2x10^{-1}$ CFU/fly on day 3; $7.6x10^{-3}$ to $0.1x10^{1}$ CFU/fly on day 4 and $9.1x10^{-2}$ to $1.1x10^{-1}$ CFU/fly) (Figure 3). Overall in this group, 58.6% flies were positive for the transconjugants. There was no statistical difference ($P>0.05$) of transfer between the DR and RD flies.

The water samples from the non-surface sterilized RD fly group were processed on a daily basis (Figure 4). There were no transconjugants on day 1. The transconjugants were found in water samples on day 2 ($7.5x10^{2}$ CFU/ml) and then again on day 3 ($1.1x10^{3}$ CFU/ml), day 4 ($8.1x10^{2}$ CFU/ml), and day 5 ($3.9x10^{2}$ CFU/ml).

In the follow-up experiment, the labella were aseptically dissected from the flies to determine the presence of transconjugants in the mouthparts. The rest of the fly was then surface sterilized to remove the surface bacteria. Then, each labellum and rest of the fly body were homogenized and processed the same way as described above. Three replicates (n=75) were done (25 flies/replicate). The concentration of donors, recipients, and transconjugants were monitored in the flies, labella, and water for five days.

The concentration of donor and recipient cells in the labellum of flies in the DR group was relatively high (~10^{3} CFU per labellum). The labella were negative for transconjugants on day 0. Transconjugants were isolated for the first time from labella of these flies on day 1 and the concentration range was from 6×10^{1} to 2.1×10^{2} CFU/labellum (Figure 5, Table 3). Five labella out of 15 were positive on day 1. The transfer frequency was $5.7x10^{-1}$ to $0.3x10^{1}$ T/D. The

transfer of transconjugants in the labella was similar for the following 3 days ($5.5x10^{-1}$ to $1.9x10^{1}$ CFU/labellum on day 2, $0.9x10^{1}$ to $4.5x10^{1}$ CFU/labellum on day 3, $0.1x10^{1}$ to $2.9x10^{1}$ CFU/labellum on day 4) (Table 3). In total, 34% of the labella were positive for transconjugants.

The concentration of donor and recipient cells in the digestive tract of the corresponding flies (surface sterilized body) in both groups (DR and RD) were not significantly different (P = 0.11, P = 0.09) from that of the non-surface sterilized flies indicating that the majority of enterococci resided in the digestive tract and not on the body surface. In the DR group, no transconjugants were detected from the fly digestive tract immediately after the exposure of donor and recipient inoculum (day 0) (Figure 6). Twenty four hours after the exposure, transconjugants appeared in the fly gut in the DR group (Figure 6, Table 4). Nine flies out of 15 were positive for transconjugants and the concentrations ranged from $3.1x10^{1}$ to $3x10^{3}$ CFU/fly. The transfer rate (T/D) ranged from $0.1x10^{1}$ to $2.6x10^{-3}$ on day 1, $8.1x10^{-3}$ to $1.1x10^{1}$ on day 2, $8.6x10^{-5}$ to $0.1x10^{1}$ on day 3 and $1.6x10^{-1}$ to $0.5x10^{1}$ CFU/fly on day 4 (Table 4). In this group of flies, 52% of the flies showed transconjugants over the four days.

In the water samples in the DR group, no transconjugants were found on the first day, after 24 hr of inoculum exposure to the flies (Figure 7). Transconjugants were isolated from water consistently with high concentration over the following days ($3.6x10^{5}$ CFU/ml on day 2; $1.1x10^{4}$ CFU/ml on day 2; $7.1x10^{2}$ CFU/ml on day 2).

In the RD group, the concentration of donor and recipient cells was lower than that in the DR group and greatly varied among flies (10^{1}-10^{4} CFU per labellum) (Figure 8). No transconjugants were detected in the labella of flies in the RD group immediately after the exposure to the inocula (Figure 8, Table 5). Only two labella (3.0%) were positive for transconjugants on day 1 with the concentration of $1.2x10^{2}$ CFU/labellum. The rate of transfer

(T/D) ranged from 5.4x 10^{-4} to 1.1x10^{-1}. No transconjugants were isolated from the labella of this group of flies on days 2, 3, and 4.

The surface sterilized group of flies in the RD group exhibited very low frequency of transfer (19%) (Figure 9, Table 10). No transfer of transconjugants occurred in the flies on day 0. The transconjugants were isolated for the first time on day 1 (Figure 9). On day 1, only 3 out of 15 flies were positive for transconjugants (range 6x10^{1} to 1.7x10^{3}). The transfer rate per donor ranged from 7.5x10^{-3} to 1.5x10^{-2} on day 1; 1.5x10^{-3} on day 2; 4x10^{-4} to 5.7x10^{-2} on day 3; 4.6x10^{-3} to 2.2x10^{-1} on day 4 (Table 10). The transfer in the flies that were fed recipient first, donor second (RD group) was significantly lower (P<0.001) than in the DR group.

Water samples from this corresponding group of flies (RD) were not positive for transconjugants on day 1. However, transconjugants were isolated from water every day for the next 3 days. The mean concentration of transconjugants was 2.4x10^{2} CFU/ml (day 2), 2.2x10^{2} CFU/ml (day 3) and 1x10^{1} CFU/ml (day 4) (Figure 10).

DISCUSSION

Bacterial evolution and rapid emergence of resistant strains emphasize the importance to explore new microenvironments that can provide the conditions for DNA transfer among bacteria (Hall, 2004; Choi and Kim, 2006; Light and Wilcks, 2006). *Enterococcus faecalis* has been recognized as a very important nosocomial pathogen (Richard et al., 2000) and for the frequent transfer of genetic determinants (Dunny et al., 1978; Murray, 1998; Simjee et al., 2006). It has been shown that the digestive tract of humans (Shoemaker et al., 2001) and animals provides the suitable conditions for the horizontal gene transfer (Dillon and Dillon, 2004; Kazimierczak and Scott, 2007). Many studies have been published that focusing on the transfer

of resistance and virulence genes of enterococci *in vivo* and *in vitro* (Jensen et al., 1998; Jensen et al., 1999; Boggard et al., 2000, Moubareck et al., 2003; Hirt et al., 2005; Lester et al., 2006; Coburn et al., 2007; Dahl et al., 2007; Jacobsen et al., 2007; Sung and Lindsay, 2007).

House flies are known as a potential mechanical vector of various bacteria, including pathogenic strains originating from animal feces and other decaying organic materials (Zurek et al., 2000; Moon, 2002). However, to my knowledge, there is only one study assessing the horizontal gene transfer in the house fly digestive tract. Petridis et al. (2006) reported recently that horizontal transfer of resistance and virulence genes can occur among the gram- negative bacteria, specifically *E. coli* strains, in the house fly gut. Chloramphenicol resistance genes on a plasmid or the lysogenic bacteriophage-born virulence gene Shiga toxin gene *stx1* (bacteriophage H-19B::Ap1) were transferred from the donor strain to the recipient strain. The rate of plasmid transfer was 10^{-2} CFU (T/D) in the house fly midgut and 10^{-3} CFU (T/D) in the crop and occurred within 1.0 hr of exposure. The authors suggested that antibiotic resistance or toxins can horizontally transfer in the fly gut by plasmid or phage transduction. There are no reports of horizontal transfer of antibiotic resistance genes or any other genes among gram-positive bacteria in the house fly gut.

In this study, horizontal transfer of antibiotic resistance genes of enterococci in the house fly gut was evaluated. For this assay, the pheromone responsive plasmid conjugation method of enterococci were followed to transfer the tetracycline resistance gene on a plasmid from the donor *E. faecalis* OGIRF:pCF10 to the recipient *E. faecalis* OGISSp strain. This transfer depends on the specific cCF10 peptide pheromone production and is restricted for the same species (Dunny et al., 1978; Buttaro et al., 2000; Hirt et al., 2005). The results clearly show that *E. faecalis* donor and recipient population established in the house fly gut within 24 hr of inoculum

exposure regardless if they received first donor or recipient inoculum. The concentration of donor, recipient, and transconjugant populations in house flies (mean concentration up to 10^5 CFU/fly) was relatively stable and similar to that obtained from field collected house flies (Chapter 2, Macovei and Zurek, 2006). Transconjugants were readily detected in the flies 24 hr after the inoculum exposure. In non-surface sterilized flies, it was not possible to determine if the transfer took place on the surface of flies or in their digestive tract. Results of the surface sterilization flies revealed that the plasmid transfer took place mostly in the gut. More importantly, the corresponding samples of drinking water were negative for transconjugants 24 hr after the inoculum exposure. The consistent presence of transconjugants in house flies at least 24 hr prior the corresponding water became positive for transconjugants clearly supports the fact that transconjugants originated from the flies. Transconjugants were also detected from the labella of mouthparts at the same time they were observed in flies. In general, the gut microenvironment provides the optimum conditions for gene transfer due to diverse microbial community and the influx of new transient microbes from food in a nutrient rich environment (Kazimierczak and Scott, 2007). Several studies that have focused on the insect gut have shown that this organ can serve as a suitable site for gene transfer (Jarrett et al., 1990; Hoffmann et al., 1998; Watanabe et al., 1998; Hinnebusch et al., 2002; Dillon and Dillon, 2004; Petridis et al., 2006) but the specific conditions of the house fly gut that allows resistance gene transfer have not been investigated. However, in house flies, the midgut has been suggested as a more favorable site for plasmid transfer than the crop (Petridis et al., 2006).

Data obtained from the house flies in this study showed a very high transfer rate of plasmids - up to 10^1T/D (range: 10^{-4} - 10^1 T/D). *Enterococcus faecalis* was extensively studied regarding the conjugative plasmids and transposons with resistance and virulence traits (Clewell

and Dunny, 2002; Gilmore et al., 2002; Coburn et al., 2007). The transfer mechanism of the plasmid pCF10 was investigated in several *in vitro* studies (Dunny et al., 1978; Buttaro et al., 2000; Clewell and Dunny, 2002; Hirt et al., 2005). Our study reports for the first time the transfer of *tet* resistance gene on pCF10 in the house fly digestive tract. Plasmid transfer rates between donor and recipient strains involving the expression of the aggregation substance were demonstrated to be as high as 10^{-1} T/D in *in vitro* studies (Dunny et al., 1978). As the sex pheromone plasmids of *E. faecalis* represent one of the most efficient conjugation mechanisms, it has been suggested that the plasmid transfer rate can exceed 10^{-1} T/D *in vivo* (Hirt et al., 2005). Transconjugant colonization level was frequently higher in this study than the donor population. It has been reported previously that plasmid transfer frequency in bacterial populations depends mainly on the donor efficiency (Dionisio et al., 2002) and the high plasmid transfer frequency is not reflected by the size of the recipient population (Turner, 2004).

From my data, it can not be concluded that all transconjugant cells originated from the conjugation itself since the transfer was monitored only every 24 hr. Consequently, the high number of transconjugants in house flies could be a result of the high plasmid transfer rate combined with the rapid growth of the transconjugant population after plasmid transfer took place. In addition, the inocula intake by flies was beyond control in this study. If individual flies were force fed with a known inoculum concentration and volume, the inoculum influx could be better controlled. Much more frequent monitoring (every hour) of the transfer rate would enable us to determine the actual transfer rate in the individual flies. Further experiments are needed to address this issue.

Interestingly, the observed transfer frequency of pCF10 was significantly higher (P = 0.0001) in the surface sterilized flies that received the donor first and the recipient second

compared to those flies that received the inoculum in the opposite order. This phenomenon is not clear and will require further studies.

It is well established that house flies harbor a diverse microbial community from their larval habitat. Adult flies are frequent visitors of decaying organic substances for feeding and/or oviposition. House flies were positive for transconjugants for the 4-5 days throughout this study period. This provides the evidence for the vector potential of house flies beyond carrying and disseminating viable bacteria with resistance genes and horizontal transfer of antibiotic resistance genes among bacteria in the digestive tract of wild house flies is possible. House flies are then capable to transmit these resistance genes by regurgitation or defecation. Previously, it was demonstrated that house flies can retain viable *E. coli* O157:H7 in the gut and labella after 3 days of exposure and these can be shed in the fly feces (Kobayashi et al., 1999; Sasaki et al., 2000).

In conclusion, house flies greatly amplify the risk of human exposure to food-borne pathogens as well as resistant strains. Our study supports the hypothesis that the house fly digestive tract provides suitable conditions for horizontal transfer of antibiotic resistance genes mediated by plasmids among gram-positive bacteria. The results of this study make a major contribution for ranking house flies far beyond a simple mechanical vector of pathogens.

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Figure 1. **Horizontal transfer of antibiotic resistance gene in non-surface sterilized house flies (N=90) (Donor first, recipient second)**

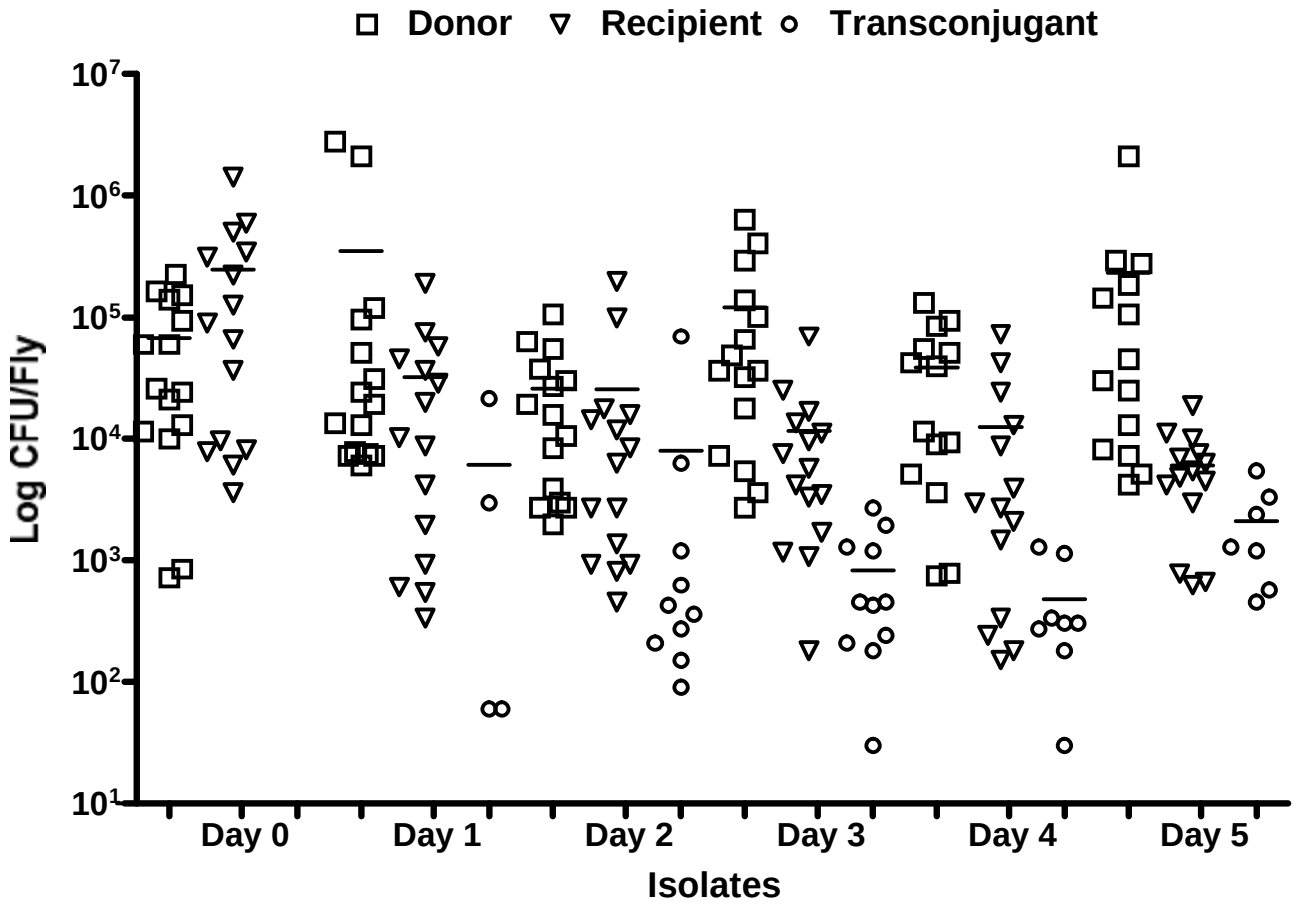
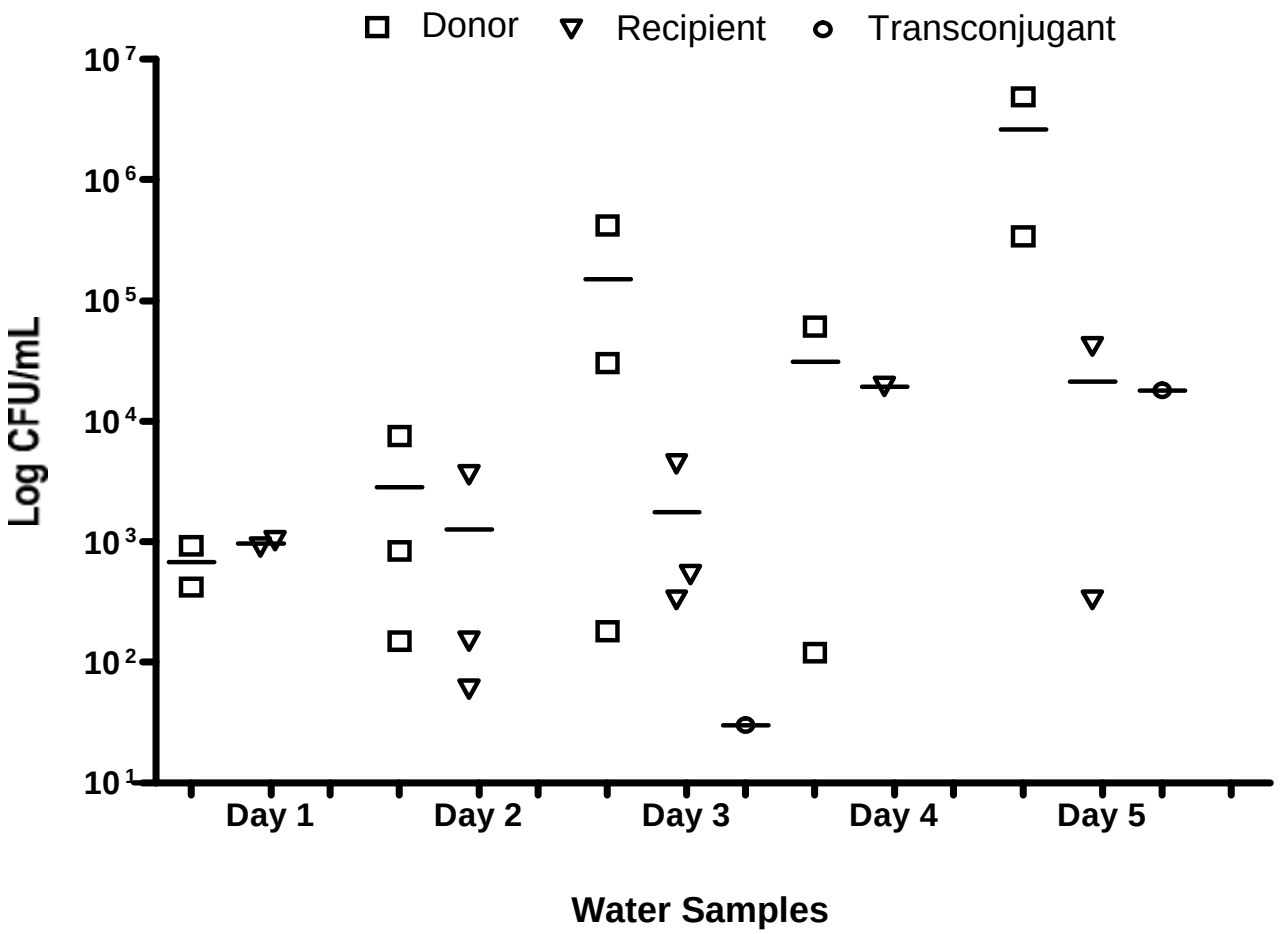


Figure 2. **Horizontal transfer of antibiotic resistance gene in water (N=90) (Donor first, recipient second)**



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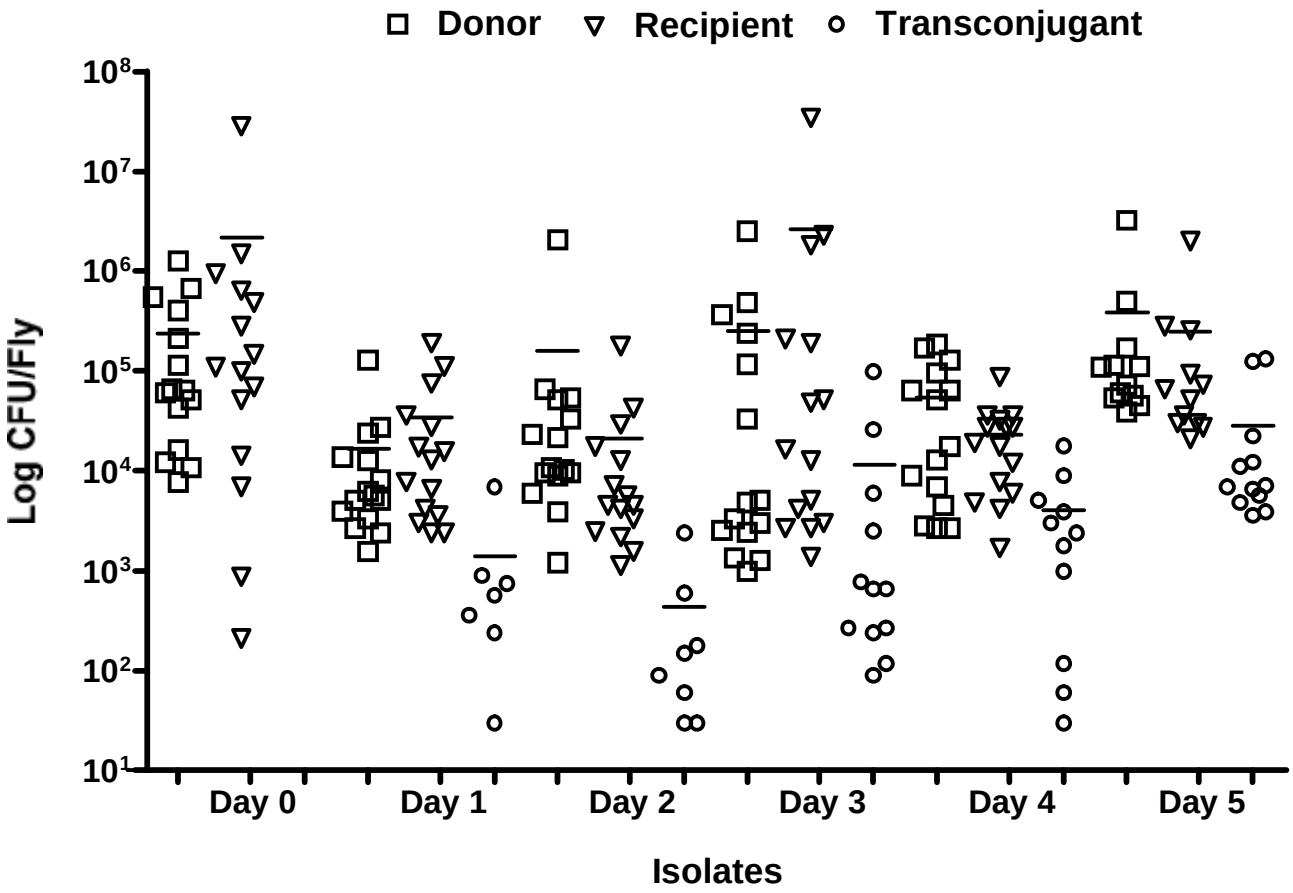


Figure 4. **Horizontal transfer of antibiotic resistance gene in water (N=90) (Recipient first, donor second)**

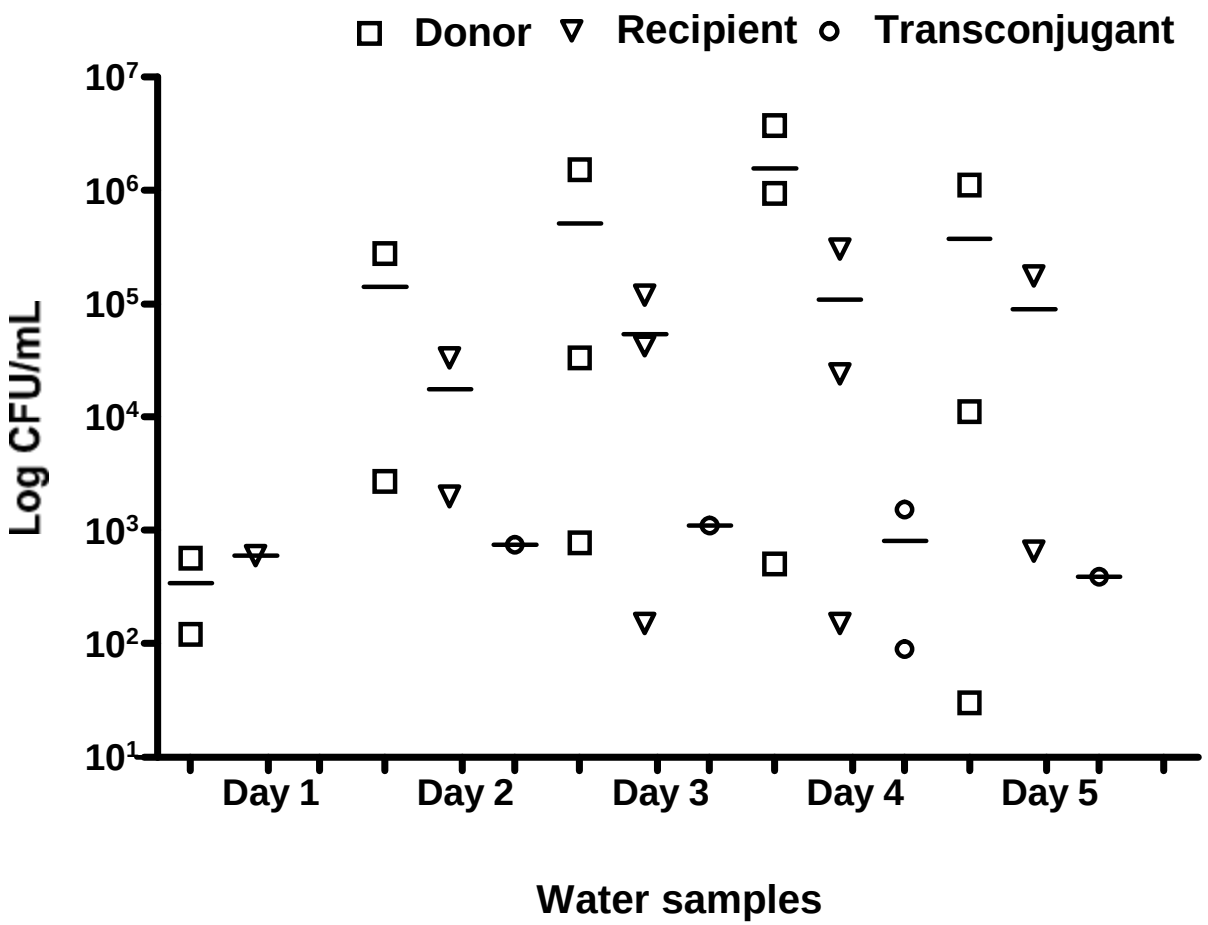


Figure 5. **Horizontal transfer of antibiotic resistance gene in the house fly labellum (N=75) (Donor first, recipient second)**

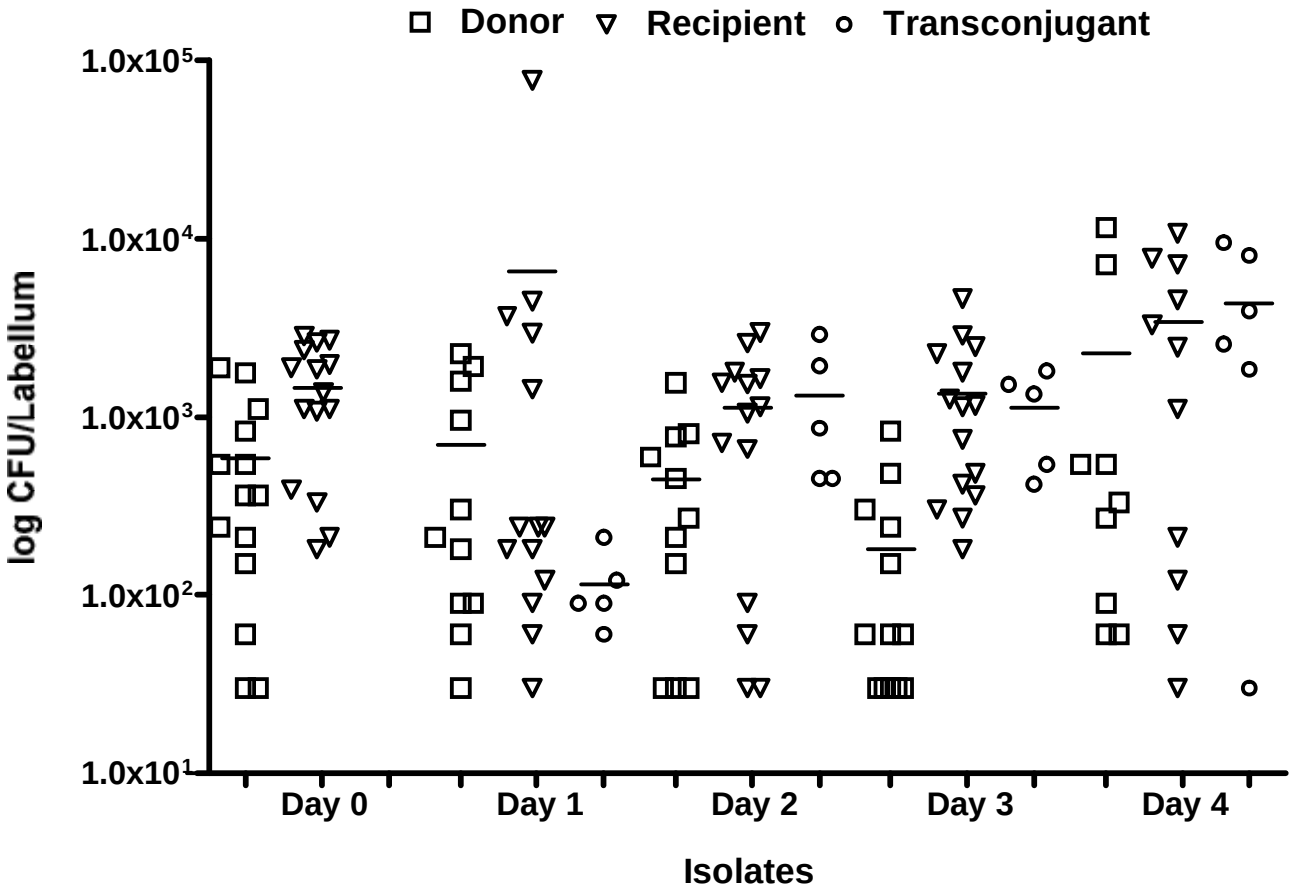


Figure 6. **Horizontal transfer of antibiotic resistance gene in surface-sterilized house flies (N=75) (Donor first, recipient second)**

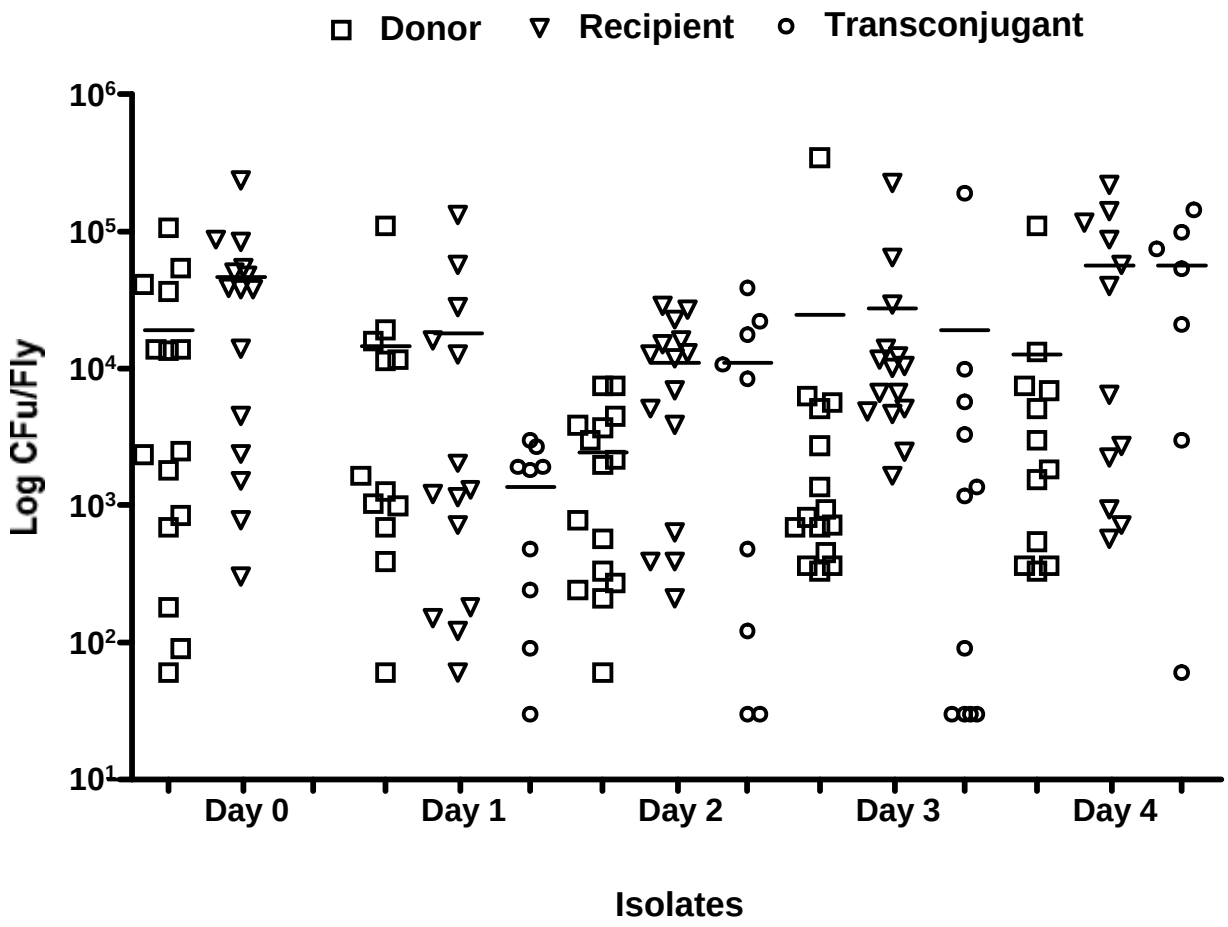


Figure 7. **Horizontal transfer of antibiotic resistance gene in water samples of surface-sterilized house flies (N=75) (Donor first, recipient second)**

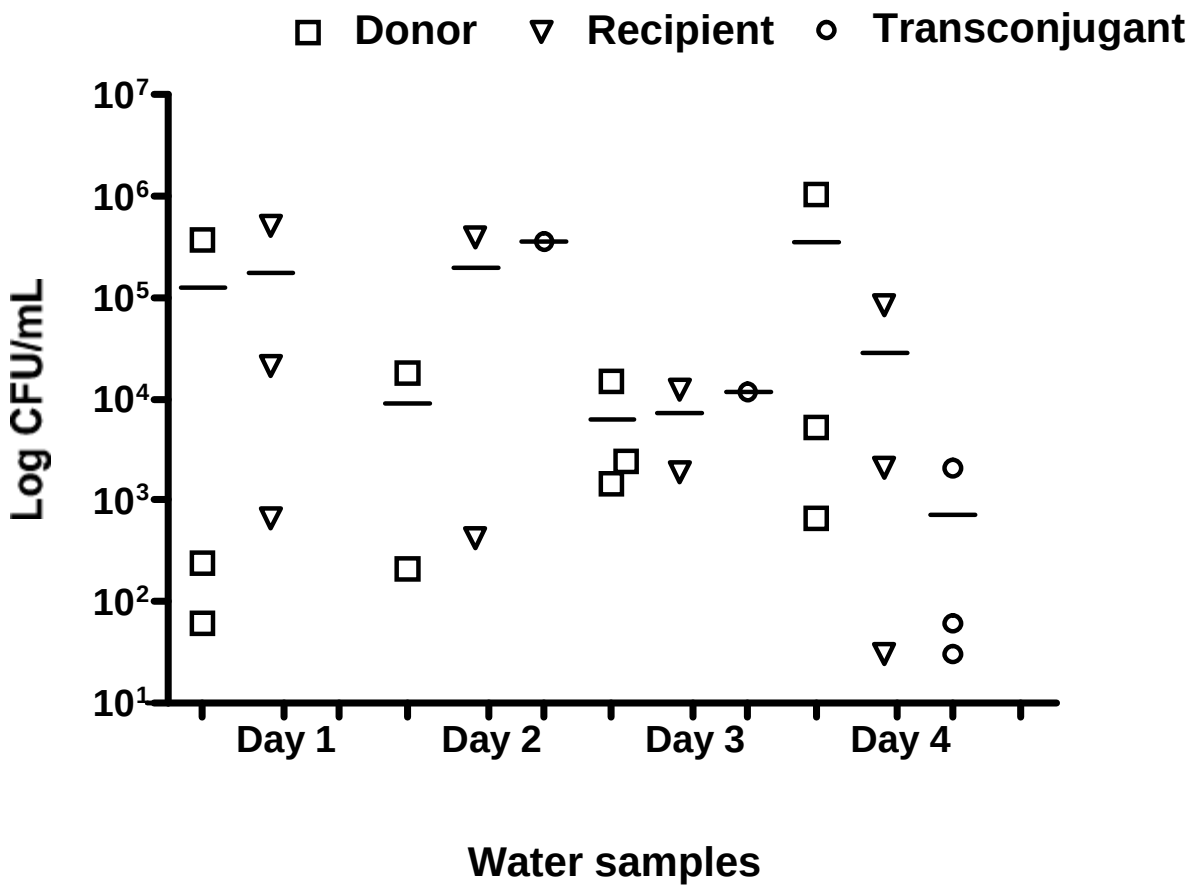


Figure 8. **Horizontal transfer of antibiotic resistance gene in surface-sterilized house fly labellum (N=75) (Recipient first, donor second)**

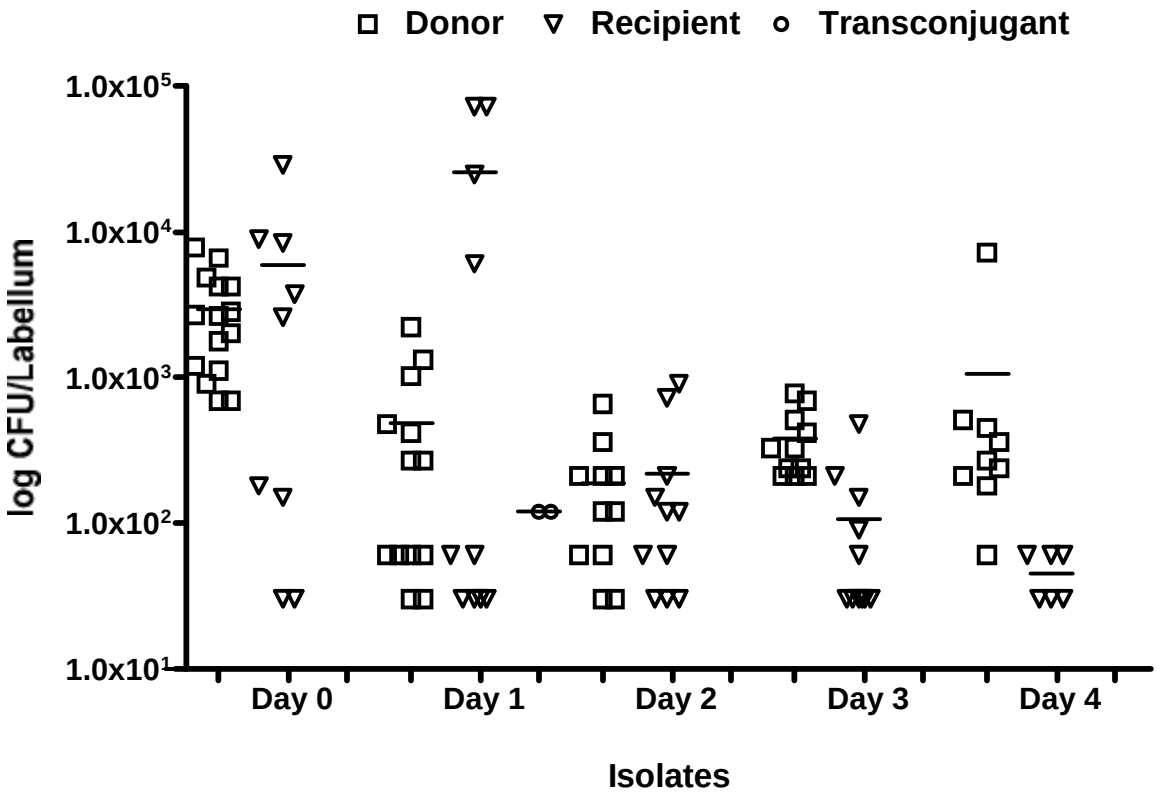


Figure 9. **Horizontal transfer of the antibiotic resistance gene in surface sterilized house flies (N=75) (Recipient first, donor second)**

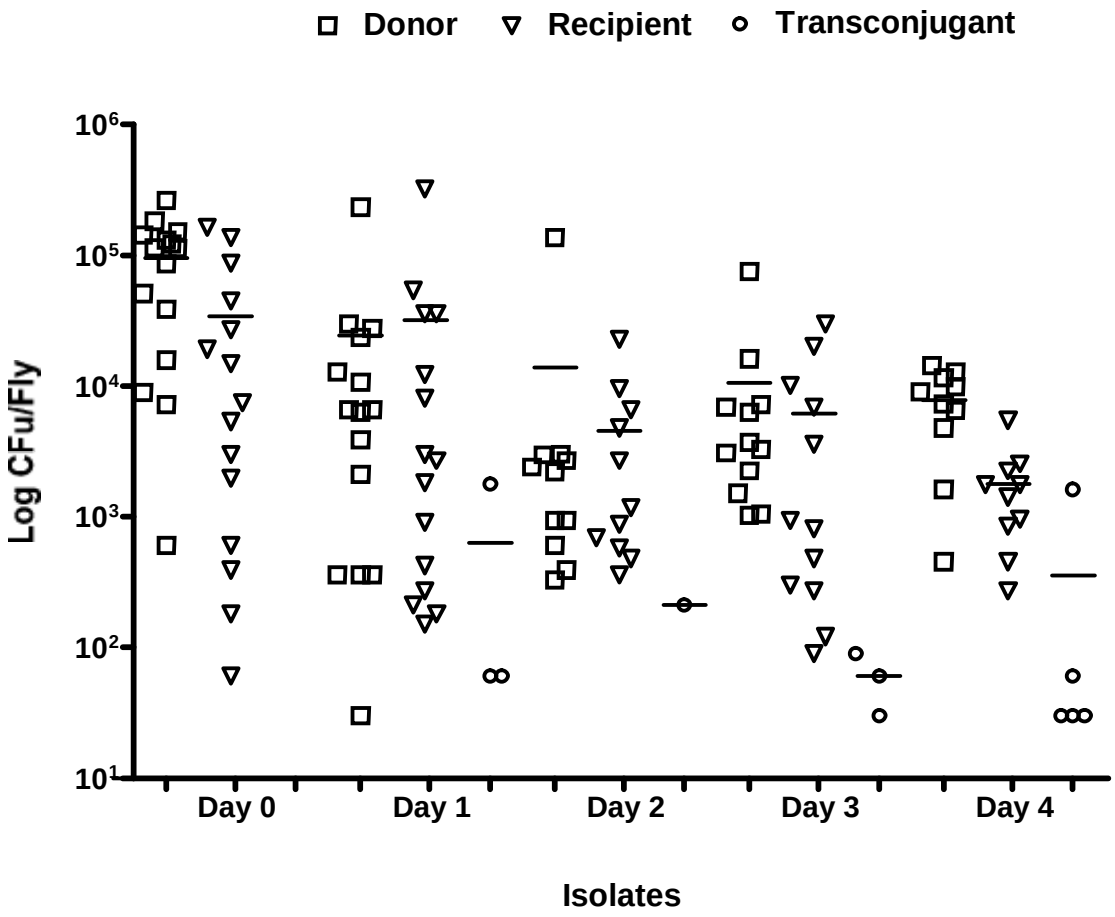
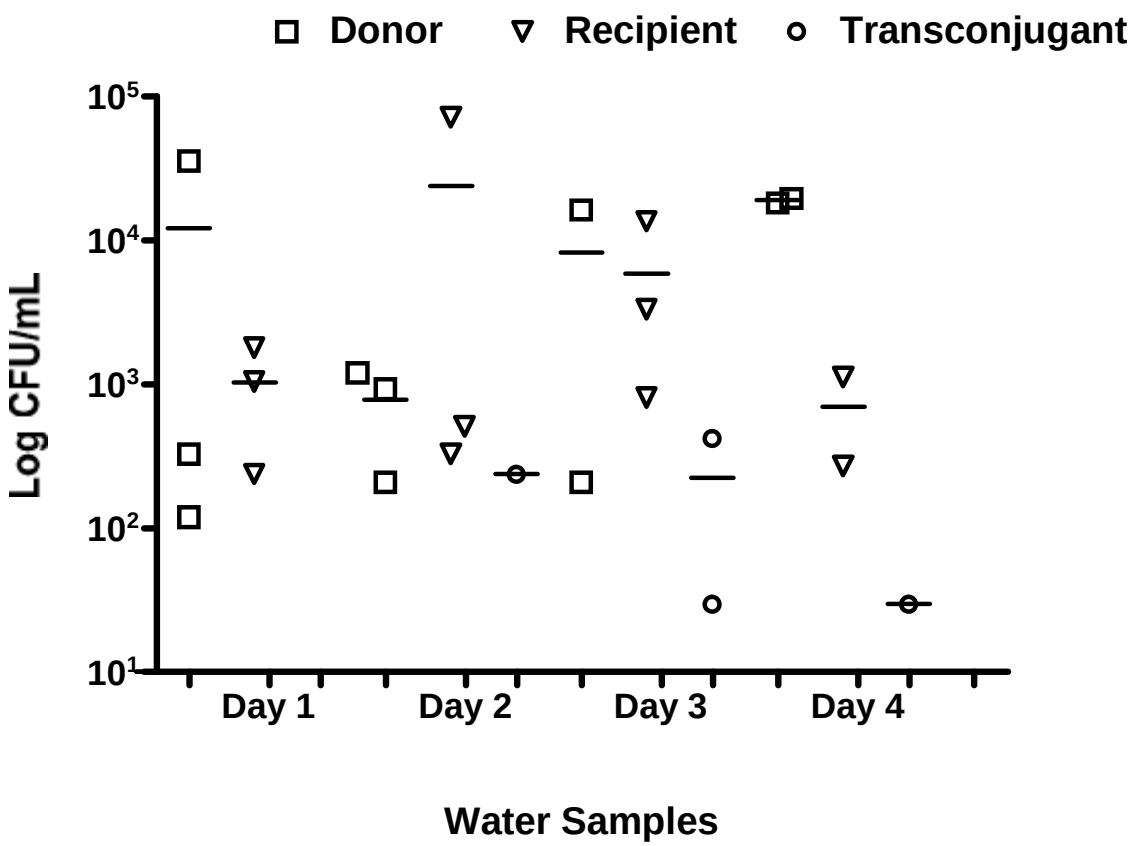


Figure 10. **Horizontal transfer of the antibiotic resistance gene in water samples of surface sterilized house flies (N=75) (Recipient first, donor second)**



CHAPTER 4

Significance and survival of enterococci during the house fly (*Musca domestica* L.) development

ABSTRACT

House flies (*Musca domestica* L.) are considered as the most important non-biting insect pest of medical and veterinary importance. House fly larvae strictly develop in decaying organic substrates that are comprised of rich and active microbial communities that are essential for house fly larval development. These microbial communities possibly provide nutrition to the house fly larvae through degradation and fermentation of organic substrates or synthesize essential nutrients (e.g. vitamins). Moreover, house fly larvae also likely ingest bacterial cells as a nutritional source. In this study, eight ATCC (American Type Culture Collection) strains of enterococci were used to evaluate the role of enterococci and their survival during the house fly development from eggs to adults. The strains used in this study included *Enterococcus avium* ATCC 14025, *E. casseliflavus* ATCC 25788, *E. durans* ATCC 19432, *E. hirae* ATCC 8043, *E. mundtii* ATCC 43186, *E. gallinarum* ATCC 49573, *E. faecalis* ATCC 19433, and *E. faecium* ATCC 19434. A total of 25 surface sterilized eggs were used for each treatment in five bioassays. Percent pupation, pupal weight, adult emergence, overall house fly survival rate, enterococcal survival rate, and concentration of enterococci in the puparium and the gut of adult flies were measured. No fly development occurred in sterile egg yolk trypticase soy agar (EYTSA) plates used as a medium for bacteria and flies. Significant difference was observed in the proportion (%) of pupation among treatments. Overall, significantly higher proportion of fly larvae reached the pupae stage when grown on *E. hirae* (80.0%) and *E. durans* (76.0%) than on other isolates. The pupal weight from EYTSA inoculated with different strains varied but was not significantly different among treatments except for *E. durans* that supported larval development into significantly less degree in this respect than *E. casseliflavus*. The highest adult emergence was observed from pupae from EYTSA with *E. hirae* (95.0%), followed by *E. avium*

(94.1%) and *E. mundtii* (92.3%). A significantly lower adult emergence was recorded on EYTSA with *E. faecium* (60.0%), *E. faecalis* (50.0%), and *E. gallinarum* (50.0%). Overall survival rate of house flies (egg to adult) was significantly higher on EYTSA with *E. hirae* (76%), *E. durans* (64.0%), *E. avium* (64%) compared to *E. gallinarum* (36.0%), *E. faecium* (33.3%), and *E. faecalis* (24.0%). Enterococci were detected in puparia and newly emerged flies with concentration ranging from 10^3-10^5 CFU/puparium and 10^2 - 10^5 CFU/fly. The prevalence of enterococci in newly emerged adults ranged from 30.8% to 89.5% and in puparia from 75.0% to 100.0%. This study indicates that potentially clinically important enterococcal strains, *E. faecalis* and *E. faecium*, did not support the fly development well comparing to commensal enterococcal strains *E. hirae*, *E. avium*, and *E. durans*.

INTRODUCTION

The house fly, *Musca domestica* L. (Diptera: Muscidae) is considered as the most common pest of great public and animal health importance. House flies have been implicated as mechanical or biological vectors of enteric pathogenic bacteria including *Salmonella* spp., *Shigella* spp. (Greenburg 1971), *Escherichia coli* O157:H7 (Kobayashi et al., 1999; Moriya et al., 1999; Sasaki et al., 2000; Alam and Zurek, 2004; Ahmad et al., 2006), *Campylobacter* spp. (Shane et al., 1985), and *Vibrio* spp. (Fotedar, 2001; Graczyk et al., 2001). In addition, they have been associated with highly virulent infections such as anthrax, ophthalmia, typhoid fever, tuberculosis, cholera and infantile diarrhea (Scott and Lettig, 1962; Greenberg, 1965; Keiding, 1986). House flies are also involved in the ecology of protozoan infections such as amoebic dysentery (Szostakowska et al., 2004); helminthic infections by pinworms, roundworms, hookworms and tapeworms (Getachew et al., 2007), as well as viral and rickettsial infections (Greenberg 1971; Gregorio, 1972; Greenberg, 1973; Graczyk, 2001). As mechanical vectors and nuisance pests, house flies represent a great public and animal health concern. Several studies have shown a positive association between the incidence of diarrheal diseases and the density of filth flies in developing countries (Esrey et al., 1991; Graczyk, 2001).

Animal manure, manure-soiled animal bedding, household garbage and other decaying organic substrates provide a suitable habitat for the growth and development of muscoid flies, primarily house flies (Zurek et al., 2000; Graczyk et al., 2001; Moon, 2002). These organic wastes comprise of diverse and active microbial communities (Schmidtmann and Martin, 1992; Zurek et al., 2000; Dillon and Dillon, 2004). Several studies addressing the significance of these microbial communities in the development of immature stages of muscoid flies including, house flies (Schmidtmann and Martin 1992; Zurek et al., 2000), stable flies (Lysyk et al., 1999;

Romero et al., 2006), horn flies (Perotti et al., 2001), and face flies (Hollis et al., 1985) have shown that larvae of these flies fail to develop on a sterile substrate, demonstrating their dependence on live bacterial communities. The principle of this symbiosis is unknown; it is possible that fly larvae require bacteria as a direct source of nutrients (Greenberg, 1954; Levinson, 1960), or these bacteria may contribute to larval absorption and metabolism of nutrients by making them more hydrolyzed. Larval survival and development of muscoid flies vary greatly depending on the bacterial species (Lysyk et al., 1999; Zurek et al., 2000; Romero et al., 2006).

The colonization of the insect gut by microbes depends on various factors such as pH, redox potential, digestive enzymes, and type of food. On the other hand, the gut microbiota also adapts to the intestinal microenvironment by changing their gene expression. Therefore, through all of these adaptations, ingested bacteria can survive in the larval midgut until pupation and establish in the alimentary tract of the newly emerged adult flies. The structural modification of pharyngeal ridges in fly maggots also supports this fact; fly larvae use this structure to filter bacteria from liquid media (Dowding, 1967; Schmidtmann and Martin, 1992; Watson et al., 1993; Zurek et al., 2000).

Enterococci are ubiquitous, Gram-positive cocci that comprise the normal flora of the intestine of animals ranging from insects (10^2 to 10^4 CFU/house fly [Macovei and Zurek, 2006]) to humans (10^5 to 10^7 CFU per gram of stool/feces [Murray, 1990]). ***Enterococcus faecalis, E. faecium*** and ***E. casseliflavus*** are enterococcal species frequently isolated from insects (Herman and Gerding, 1991; Jhonston and Jaykus, 2004; Sherer et al., 2005; Macovei and Zurek, 2006). Some enterococcal species are opportunistic pathogens and recognized as the third most frequent cause of nosocomial septicemia in the USA (Wisplinghoff et al., 2004). Among all the species,

E. faecium and *E. faecalis* are clinically the most important species and comprise up to 80% and 20% of clinical isolates, respectively (Huycke et al., 1998; Reynolds et al., 2004; Coque et al., 2005). Enterococci have the ability to acquire antibiotic resistance genes and can transfer these genes through plasmids and transposons to more pathogenic bacteria such as *Staphylococcus aureus* (Weigel et al., 2003). In the USA, 28.5% of nosocomial infections in hospitals are caused by vancomycin resistant enterococci (VRE) (CDC, 2002; NNIS report, 2004; Sherer et al., 2005) and these infections are very difficult to control.

The objective of this study was to evaluate the symbiotic relationship between enterococci and house flies in terms of nutrition for larval development as well as survival of enterococci during house fly development and enterococcal colonization of the gut of adult flies.

RATIONALE

This is the first study evaluating the role of enterococci in growth and development of house flies. As a ubiquitous commensal and opportunistic pathogen, it is important to identify the fate of enterococci throughout the developmental stages of the house fly, a common pest of public and health importance. This study will provide the basis for understanding *Enterococcus* and house fly symbiosis and contribution of different enterococcal strains including potentially pathogenic strains to house fly larval development. This study will also evaluate the vector potential of house flies for the dissemination of enterococci in the environment.

OBJECTIVES

1. To assess the development of house flies reared on an artificial medium with different species of enterococci.
2. To examine the survival of enterococci during house fly development**.**

HYPOTHESIS

Enterococci support the development of house flies, survive pupation, and colonize the gut of adult flies.

MATERIALS AND METHODS

Enterococcal strains: Eight different species of enterococci (type strains from ATCC: American Type Culture Collection) were used in these experiments: *Enterococcus avium* ATCC 14025, *E. casseliflavus* ATCC 25788, *E. durans* ATCC 19432, *E. hirae* ATCC 8043, *E. mundtii* ATCC 43186, *E. gallinarum* ATCC 49573, *E. faecalis* ATCC 19433, *E. faecium* ATCC 19434.

Preparation of EYTSA (Egg yolk trypticase soy agar) media: Trypticase soy broth agar (Difco) with egg yolk was prepared as described in previous studies (Watson et al., 1993; Zurek et al., 2000). Briefly, trypticase soy broth (Difco), with agar (12g/L) (Difco), was autoclaved and left in a water bath to cool down to 51-55^{o}C. Large commercial chicken eggs (2 egg yolks/l) were surface-sterilized by submerging into the 90% ethanol for one hour. When the TSBA medium was about 51-55^{o}C, yolks were aseptically separated from the rest of the egg and mixed with the medium. The medium was poured into deep Petri dishes (100 x 25 mm) and cooled down.

Surface sterilization of house fly eggs: Freshly laid house fly eggs were harvested from the laboratory house fly colony, Department of Entomology**,** Kansas State University. Eggs were collected to a sterile 15 ml falcon tube and immediately surface sterilized. Surface sterilization technique was followed standard procedures described in previous studies (Zurek et al., 2001). Briefly, eggs were washed with 10% bleach solution (0.05% sodium hypochlorite) for one minute. After pouring out bleach, eggs were rinsed with sterile de-ionized water three times. Eggs were then washed with 70% ethanol solution for one minute followed by rinsing with autoclaved de-ionized water three times. After surface sterilization, eggs were transferred aseptically on a sterile moist black filter paper to sterile Petri dishes. Petri dishes were incubated at 28^{o}C for 24 hr to allow the eggs to hatch.

Bacteria on EYTSBA medium: Bacterial suspensions were made for eight different enterococcal ATCC strains from fresh cultures and then streaked with a sterile swab stick on EYTSBA plates covering approximately 3/4 of the plate. Approximately ¼ of the plate was left sterile on every plate for placement of fly larvae. All plates were incubated at 37^{o} C for 18 hours.

Introduction of 1st-instar larvae: After 24 hr, when the eggs hatched, fly larvae were transferred aseptically with a sterile brush to the open space of the EYTSBA plates with each *Enterococcus* ATCC strain. Each bioassay was conducted with 5 larvae/plate/*Enterococcus* ATCC strain. All bioassays were replicated five times. For control group, sterile larvae were transferred to EYTSBA plates without any bacterial inoculation. All plates were incubated at 28^{o}C till pupation.

Monitoring fly survival rate: All plates were examined daily for larval mortality and pupation. As soon as the larva pupated, the pupae were removed from the plates, weighed and surface sterilized. All pupae were transferred to individual sterile Petri dishes and incubated at 28^{o}C for adult emergence. The pupation rate, adult emergence and survival rate, pupal weight, and adult weight were recorded.

Determination of enterococci in puparia and adult house flies: After emergence, all flies and puparia were homogenized in Phosphate Buffer Saline solution (1 ml/ sample). Two 10-fold serial dilutions were made and drop plated on m-ENT agar plates to count enterococcal population. Enterococci were confirmed phenotypically as described previously (Chapter 2).

Statistical analysis: Data for percent pupation, fly emergence, fly survival (egg to adult), and survival of enterococci in fly adults and puparia were transformed with arcsine square root [arcsine sqrt (percent/100)] to stabilize error variance (Gomez and Gomez 1984) and analyzed using analysis of variance (ANOVA). Means were compared by the least-squares means (LSMEANS) protocol (P=0.05) of the general linear model (PROC GLM) (SAS Institute 2003). Although all tests of significance (except pupal weight) were based on the transformed data, the untransformed percent values are reported.

RESULTS

Our bioassays using artificial medium EYTSBA inoculated with eight different ATCC enterococcal strains demonstrated that fly larvae failed to develop on the sterile media and that enterococci are required to complete larval development. Pupation occurred at almost the same time in all media plates inoculated with different ATCC strains and no significant differences occurred among treatments in length of time for fly larvae reaching pupation (data not shown). Overall, a significantly higher proportion of fly larvae reached the pupal stage when kept on media inoculated with *E. hirae* compared to that of *E. mundtii* (P = 0.0014), *E. faecalis* (P = 0.0004), and *E. faecium* (P = 0.0176) (Table 1). The proportion of larval pupation on media inoculated with *E. hirae* (80.0%) did not differ significantly from that on media inoculated with *E. durans* (76.0%, P = 0.6266), *E. gallinarum* (72.0%, P = 0.3181), *E. avium* (68.0%, P = 0.1420) and *E. casseliflavus* (68.0%, P = 0.1420) (Table 1). A significantly lower proportion of pupation was observed on media inoculated with the potentially human pathogenic strain, *E. faecalis* (48.0%) compared to that of all other strains, except *E. faecium* (P = 0.1461) and *E. mundtii* (P = 0.6266) (Table 1).

The mean pupal weight did not differ significantly among *E. avium*, *E. gallinarum*, *E. durans*, *E. hirae*, *E. mundtii*, *E. faecalis*, *E. faecium* and ranged from 0.018 to 0.020 g (Table 1). Only fly larvae fed on media inoculated with *E. casseliflavus* had a significantly greater pupal weight that those fed on *E. durans* (P = 0.0117).

Regardless of the strain, house flies started to emerge in 3-4 days after pupation. The proportion (%) of adult emergence was significantly higher in larvae fed on EYTSA inoculated with *E. hirae* (95.0%, P = 0.0003, 0.0013, 0.0002), *E. avium* (94.1%, P = 0.0003, 0.0013, 0.0002), *E. mundtii* (92.3%, P = 0.0007, 0.0026, 0.0003) and *E. durans* (84.2%, P = 0.0052,

0.0173, 0.0025) compared to *E. faecalis* (50.0%), *E. faecium* (60.0%), and *E. gallinarum* (50.0%). The adult emergence on EYTSA inoculated with *E. hirae* did not differ significantly from that on media inoculated with *E. avium* (94.1%, $P = 1$), *E. mundtii* (92.3%, $P = 0.8$), and *E. durans* (84.2%, $P = 0.3228$) (Table 1).

The overall survival rate of house fly larvae (egg to adult) varied among treatments. The highest adult emergence was recorded from the media inoculated with *E. hirae* (76.0%), followed by EYTSA inoculated with *E. avium* (64.0%) and *E. durans* (64.0%). A significantly lower proportion of adult emergence was recorded in media with *E. faecalis* (24.0%) compared to *E. hirae* ($P < 0.0001$), *E. avium* ($P < 0.0001$), *E. mundtii* ($P = 0.0013$) and *E. durans* ($P < 0.0001$) and *E. casseliflavus* ($P = 0.0013$) treatments (Table 1).

Enterococci were detected in newly emerged fly adults that were fed as larvae on different enterococcal strains. Table 1 shows the prevalence of enterococci in newly emerged adults that ranged from 25.0 to 89.5%. The highest colonization was recorded in *E. hirae* (89.5%) followed by *E. durans* (87.5%) in the gut of newly emerged flies and that was significantly different ($P < 0.0001$ and $P < 0.01$) compared to all other strains. The poorest colonization rate was observed with *E. casseliflavus* (25.0%). The bacterial concentration ranged from 10^2 to 10^5 CFU/fly. The concentration varied among the individual flies and showed high standard deviations. *E. gallinarum* survived in the fly gut throughout the developmental time with the highest mean concentration of $6.6 \pm 5.7 \times 10^5$ CFU/fly (Table 1).

The puparia were also tested for the presence of enterococci. The majority of puparia (75%- 100%) were positive for enterococci. Hundred percent of puparia were positive from EYTSA with *E. hirae, E. faecalis, E. gallinarum, E. mundtii* (Table 1). The enterococcal

concentration was found higher in the puparia than the corresponding fly gut (Table 1). The mean concentration range among different strains was 10^3-10^5 CFU/puparium.

DISCUSSION

House fly (*Musca domestica* L.) larvae develop primarily in decaying organic substrates such as animal manure, household garbage, and other decaying organic materials (Moon et al., 2001). This type of habitat contains a rich microbiota that likely provides essential nutrients for larval development through continuous fermentation or degrading organic substrate (Zurek et al., 2000; Moon et al., 2001; Moon, 2002). Developing and residing in these sites with diverse microbes make house flies a likely vector for bacteria from feces/manure including human and animal pathogens (Sasaki et al., 2000; Graczyk et al., 2001). Several studies have shown the symbiotic relationship between microbes and muscoid flies in terms of nutrition for larval development (Schmidtmann and Martin, 1992; Zurek et al., 2000), bacterial digestion in the fly gut, oviposition stimuli, and vector competence (Espinosa-Fuentes and Terra, 1987; Zurek et al., 2000; Schmidtmann and Martin, 2002; Petridis et al., 2006; Romero et al., 2006).

In this study, we investigated the role of enterococci in the house fly larval development as well as their survival throughout the fly development from eggs to adults. To our knowledge, no previous work has been done for *Enterococcus*-house fly symbiosis in terms of support of insect development. Our bioassays using the artificial medium (EYTSBA) inoculated with eight different ATCC enterococcal strains demonstrated that fly larvae failed to develop on the sterile media and bacteria are required to complete larval development. This is in agreement with results of similar studies with muscoid fly larvae and different enteric bacteria showing strict dependence of larval development on live and active bacterial cells (Schmidtmann and Martin,

1992; Zurek et al., 2000). However, the overall fly survival rate from eggs to adults varied depending on the bacterial strain. The highest fly survival was observed with *E. hirae* (76%) compared to potentially pathogenic strains, *E. faecalis* (24%) and *E. faecium* (36%). Likewise, percent pupation (80%), percent adult emergence (95%) was significantly higher in larvae fed on YETSA inoculated with *E. hirae* compared to that of *E. faecalis* (48% and 50% respectively) and *E. faecium* (60% and 60% respectively). *E. faecalis* and *E. faecium* are opportunistic pathogens and clinically most important whereas *E. hirae* is categorized more as a commensal strain. In the USA, 60-80% of nosocomial infections by enterococci are caused by *E. faecalis* strains (Sherer et al., 2005). Other studies with potentially pathogenic strains also showed low survival rate for house fly larval development (Zurek et al., 2000).

In nature, house flies receive nutrition from complex interactions of the diverse bacterial community. Larvae develop in decaying organic substrates with microbes and they have the capacity to digest bacteria for nutrition. Bacteria in the house fly midgut are lysed by several proteases (lysozymes, cathepsin) and low pH (Espinosa-Fuentes and Terra, 1987; Dillon and Dillon, 2004). Intestinal commensals act in food digestion and provide micronutrients by degrading complex molecules. Pupal weight data indicated that house fly larvae received enough nutrients to gain sufficient weight mainly with *E. hirae*. *Enterococcus hirae* and *E. durans* also showed the highest colonization rate (89.5%) in the gut of newly emerged flies compared to that of all other strains. *E. faecalis* did not support larval development well and it was recovered from the gut of 50% of newly emerged adult flies only. This fact indicates that potentially human pathogenic strains do not have the capacity to adapt, survive pupation, and establish in this microenvironment. In addition, the virulence factors of *E. faecalis* and *E. faecium* including gelatinase, aggregation substance, cytolysin (Clewell, 1993; Hancock and Gilmore, 2000;

Hancock and Perego, 2004) might have a negative impact on fly tissues and overall biology resulting in a low survival rate of house flies although additional studies will be required to fully address this phenomena.

Interestingly, not all newly emerged adult flies were positive for enterococci in the digestive tract. In contrast, the majority (75 - 100%) of the puparia was positive for enterococci indicating that many bacteria are left behind in the puparium when the adult fly emerges. The bacterial concentration in the fly gut ranged from 10^2 to 10^5 CFU/ fly and the mean concentration range among different enterococcal strains ranged from 10^3 to 10^5 CFU/puparium. Other studies have also confirmed the fact that fly maggots were carrying pathogenic strains in their gut when reared on substrates inoculated with *T. gondii* (Wallace, 1971) or *C. parvum* (Graczyk et al., 1999) but no pathogens were isolated from the gut of newly emerged flies. During the complete metamorphosis, the midgut of the adult insect is completely reformed from larval stage by multiplication of regenerative cells. These regenerative cells eventually form the lining of the lumen of the new alimentary canal for the next stages of life cycle such as pupa or adult (Chapman, 1998).

Clearly, enterococcal cells are able to survive the pupation process and can colonize the puparium and the gut of adult flies. Further research will be necessary to elucidate how the bacterial cells escape the enzymatic processes during metamorphosis and/or how the fly larva/pupa avoids bacteremia. Great differences were observed in colonization of the digestive tract of adult flies (25 - 90% prevalence). These finding have a great relevance for the vector capacity of adult house flies to transmit bacteria originating from animal manure. The bacteria that survive pupation and colonize the adult gut first likely establish the resident gut bacterial

community and are consequently disseminated to the environment in the house fly flight/dispersal range.

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Table 1. Contribution and survival of enterococci in the gastro-intestinal tract during the house fly development (egg to adult) on EYTSA (egg yolk trypticase soy agar) medium (n =25)

ATCC strains	Pupae wt.(g) Mean ± SD	Percent pupation	Percent adult emergence	Percent survival (egg to adult)	Percent adult positive for enterococci	Percent puparium positive for enterococci	Enterococci (CFU/ml) Mean ± SD	
							Adult fly	Puparium
E. avium	0.019 ± 0.003 ^{ab}	68 ^{abc}	94.11 ^{a}	64 ^{a}	37.5 ^{c}	88.89 ^{b}	$5.9 \pm 8.2 \times 10^{2}$	$1.2 \pm 2.6 \times 10^{5}$
E. casseliflavus	0.021 ± 0.002 ^{a}	68 ^{abc}	70.58 ^{bc}	48 ^{b}	25 ^{c}	85.71 ^{cd}	$7.7 \pm 9.4 \times 10^{2}$	$1.4 \pm 1.6 \times 10^{3}$
E. durans	0.018 ± 0.008 ^{b}	76 ^{ab}	84.21 ^{ab}	64 ^{a}	87.5 ^{b}	88.89 ^{b}	$0.7 \pm 2.6 \times 10^{5}$	$2.7 \pm 3 \times 10^{3}$
E. hirae	0.020 ± 0.004 ^{ab}	80 ^{a}	95 ^{a}	76 ^{a}	89.47 ^{a}	100 ^{a}	$0.6 \pm 2.4 \times 10^{5}$	$1.8 \pm 3.6 \times 10^{5}$
E. mundtii	0.019 ± 0.003 ^{ab}	52 ^{cd}	92.31 ^{ab}	52 ^{b}	30.77 ^{c}	100 ^{bc}	$3.0 \pm 3.2 \times 10^{2}$	$5.3 \pm 5.7 \times 10^{3}$
E. gallinarum	0.019 ± 0.036 ^{ab}	72 ^{ab}	50 ^{d}	36 ^{bc}	33.33 ^{c}	100 ^{cde}	$6.6 \pm 5.7 \times 10^{5}$	$4.7 \pm 2.6 \times 10^{3}$
E. faecalis	0.020 ± 0.002 ^{ab}	48 ^{d}	50 ^{cd}	24 ^{c}	50 ^{c}	100 ^{de}	$0.6 \pm 1.0 \times 10^{4}$	$1.1 \pm .08 \times 10^{3}$
E. faecium	0.019 ± 0.002 ^{ab}	60 ^{bcd}	60 ^{cd}	36 ^{bc}	33.33 ^{c}	75 ^{de}	$1.1 \pm 1.3 \times 10^{2}$	$1.6 \pm 3.7 \times 10^{5}$

*Values within same column followed by the same letter are not significantly different (p> 0.05; PROC GLM; SAS Institute 2003)

Appendix A

Chapter 3- Tables

Table 1. Horizontal transfer of the antibiotic resistance gene in the non surface-sterilized house flies (donor first, recipient second)

Fly #	Day 0				Day 1				Day2				Day 3				Day 4				Day 5			
	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D
1	$7.2x10^2$	$3.6x10^4$	0	-	$7.2x10^3$	$3.3x10^2$	0	-	$8.4x10^3$	$4.5x10^2$	$2.7x10^2$	$3.2x10^{-2}$	$3.6x10^4$	$3.4x10^3$	$1.9x10^3$	$5.4x10^{-2}$	$4.2x10^4$	$3.3x10^2$	0	-	$2.9x10^5$	$6.6x10^2$	0	-
2	$1.6x10^5$	$7.8x10^3$	0	-	$2.4x10^4$	$9.3x10^2$	0	0	$6.3x10^4$	$1.3x10^3$	$2.1x10^2$	$3.3x10^{-3}$	$1.0x10^5$	$1.2x10^3$	$4.5x10^2$	$4.4x10^{-3}$	$5.4x10^4$	$1.8x10^2$	0	-	$1.4x10^5$	$7.8x10^2$	0	-
3	$8.4x10^3$	$3.6x10^5$	0	-	$7.2x10^3$	$6x10^2$	0	0	$1x10^4$	$8.4x10^3$	$6.3x10^3$	$6x10^{-1}$	$4.8x10^4$	$4.2x10^3$	$4.2x10^2$	$8.7x10^{-3}$	$7.5x10^2$	$1.5x10^2$	0	-	$1.8x10^5$	$6.3x10^2$	0	-
4	$1.2x10^4$	$9.6x10^5$	0	-	$1.2x10^4$	$5.4x10^2$	0	0	$3.7x10^4$	$2.7x10^3$	$9x10^1$	$2.4x10^{-3}$	$2.9x10^5$	$2.4x10^4$	$1.2x10^3$	$4.1x10^{-3}$	$5.1x10^4$	$3.9x10^3$	$2.7x10^2$	$5.2x10^{-1}$	$2.1x10^6$	$1.1x10^4$	$5.4x10^3$	$2.5x10^{-3}$
5	$9.9x10^3$	$6x10^3$	0	-	$1.2x10^5$	$1.9x10^3$	0	0	$1x10^5$	$9.3x10^2$	$4.2x10^2$	$4x10^{-3}$	$3.2x10^4$	$1.8x10^2$	$3x10^1$	$9.3x10^{-4}$	$9.3x10^4$	$2.4x10^2$	0	-	$2.7x10^5$	$4.2x10^3$	$2.4x10^3$	$8.6x10^{-3}$
6	$1.1x10^4$	$8.1x10^3$	0	-	$2.0x10^6$	$1.9x10^4$	$3x10^3$	$1.4x10^{-3}$	$5.4x10^4$	$6.3x10^3$	0	-	$6.3x10^5$	$6.9x10^4$	$2.7x10^3$	$4.2x10^{-3}$	$1.1x10^4$	$1.4x10^3$	$1.1x10^3$	$1x10^{-1}$	$1x10^5$	$3x10^3$	$5.7x10^2$	$5.4x10^{-3}$
7	2.5x	$2.2x10^5$	0	-	$2.7x10^6$	$8.7x10^2$	$2.1x10^1$	$7.6x10^{-3}$	$1.9x10^2$	$9.3x10^2$	0	-	$6.6x10^4$	$7.5x10^1$	$1.2x10^3$	$1.9x10^{-3}$	$1.3x10^5$	$8.7x10^3$	$3x10^1$	$2.2x10^{-4}$	$8.1x10^3$	$7.5x10^3$	$1.2x10^5$	$1.5x10^{-1}$
8	$2.4x10^4$	$1.2x10^5$	0	-	$1.9x10^4$	$5.7x10^4$	$6x10^1$	$3.1x10^{-3}$	$3x10^4$	$2.7x10^3$	0	-	$1.7x10^4$	$1x10^3$	$4.5x10^2$	$2.5x10^{-2}$	$9.3x10^3$	$3x10^3$	$3.3x10^2$	$3.5x10^{-2}$	$5.1x10^3$	$5.4x10^3$	$4.5x10^2$	$8.8x10^{-2}$
9	$2.1x10^4$	$3.4x10^5$	0	-	$9.6x10^4$	$4.2x10^3$	0	-	$3x10^3$	$8.1x10^2$	0	-	$3.6x10^4$	$1.7x10^3$	$2.4x10^2$	$6.6x10^{-3}$	$3.6x10^3$	$2.1x10^3$	0	-	$4.5x10^4$	$1.8x10^4$	$1.2x10^3$	$2.6x10^{-2}$
10	$1.4x10^5$	$9x10^4$	0	-	$3.0x10^4$	$1x10^4$	0	-	$1.9x10^4$	$1.9x10^5$	$6.9x10^4$	$0.3x10^3$	$1.3x10^5$	$3.3x10^3$	$1.8x10^2$	$1.3x10^{-3}$	$8.4x10^4$	$2.7x10^3$	0	-	$4.2x10^3$	$6.9x10^3$	$3.3x10^3$	$7.8x10^{-1}$
11	$6x10^4$	$3.1x10^5$	0	-	$1.3x10^4$	$4.5x10^4$	0	-	$2.7x10^4$	$9.9x10^4$	$1.2x10^3$	$4.4x10^{-2}$	$3.6x10^3$	$5.7x10^3$	0	-	$9x10^3$	$1.2x10^4$	$1.2x10^3$	$1.4x10^{-1}$	$3x10^4$	$4.5x10^3$	0	-
12	$9.3x10^4$	$5x10^5$	0	-	$7.8x10^3$	$3.6x10^4$	0	-	$1.5x10^4$	$1.7x10^4$	$1.5x10^2$	$9.6x10^{-3}$	$7.2x10^3$	$1.1x10^4$	0	-	$3.9x10^4$	$7.2x10^4$	$3x10^2$	$7.6x10^{-3}$	$2.4x10^4$	$6.3x10^3$	0	-
13	$1.5x10^5$	$5.9x10^5$	0	-	$5.1x10^4$	$7.5x10^4$	0	-	$3.9x10^3$	$1.1x10^4$	$6.3x10^2$	$1.6x10^{-1}$	$5.4x10^3$	$1.3x10^4$	0	-	$5.1x10^3$	$2.4x10^4$	$3x10^2$	$5.8x10^{-2}$	$7.2x10^3$	$9.9x10^3$	0	-
14	$2.2x10^5$	$1.4x10^6$	0	-	$7.5x10^3$	$2.8x10^4$	$6x10^1$	$8x10^{-3}$	$2.7x10^3$	$1.4x10^4$	$3.6x10^3$	$1.3x10^{-1}$	$2.7x10^3$	$9.6x10^3$	0	-	$7.8x10^2$	$4.2x10^4$	$1.8x10^2$	$2.3x10^{-1}$	$1.2x10^4$	$4.8x10^3$	0	-
15	$6x10^4$	$6.6x10^4$	0		$6x10^3$	$1.8x10^5$	0	-	$2.7x10^3$	$1.5x10^4$	0	-	$4.0x10^5$	$1.6x10^4$	$2.1x10^2$	$5.2x10^{-4}$	-	-	-	-	-	-	-	-

Table 2. Horizontal transfer of the antibiotic resistance gene in the non surface-sterilized house flies (recipient first, donor second)

Fly #	Day 0				Day 1				Day2				Day 3				Day 4				Day 5			
	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D
1	1x10^4	3.6x10^4	0	-	5.1x10^3	3.6x10^3	0	-	1.2x10^5	1.5x10^3	1.5x10^2	1.2x10^-1	4.7x10^5	4.8x10^4	6.6x10^2	1.3x10^-3	1.2x10^4	2.7x10^4	1.7x10^4	0.1x10^1	1.1x10^5	2.5x10^5	5.7x10^5	5x10^-2
2	6.6x10^4	8.7x10^2	0	-	2.7x10^5	4.2x10^3	0	-	9x10^3	4.5x10^3	6x10^1	6.6x10^-3	3.6x10^5	1.7x10^6	6x10^-3	1.6x10^-2	1.7x10^4	3.6x10^4	1.8x10^5	1x10^-1	5.4x10^4	3x10^4	3.9x10^5	7.2x10^-2
3	4.2x10^4	1.4x10^4	0	-	1.5x10^5	2.4x10^3	0	-	5.1x10^4	5.7x10^3	0	-	2.3x10^5	2.1x10^5	7.8x10^2	3.2x10^-3	4.5x10^3	6x10^3	3x10^3	6.6x10^-1	1.6x10^5	7.2x10^4	2.2x10^4	1.3x10^-1
4	6.3x10^4	6.9x10^3	0	-	3.9x10^3	3x10^3	0	-	1.0x10^4	2.4x10^3	0	-	2.4x10^6	2.2x10^6	9.9x10^4	3.9x10^-2	6.3x10^4	3.6x10^4	0	-	1.0x10^5	3x10^4	7.2x10^5	6.6x10^-2
5	6x10^4	2.1x10^2	0	-	2.4x10^4	1.1x10^5	0	-	6x10^3	4.5x10^3	0	-	3.3x10^4	3.4x10^7	2.5x10^4	7.8x10^-1	5.1x10^4	1.7x10^4	0	-	6x10^4	5.1x10^4	1.1x10^4	1.8x10^-1
6	7.8x10^3	2.7x10^5	0	-	2.4x10^5	1.8x10^5	9x10^2	3.7x10^-1	2x10^6	4.2x10^4	6x10^2	2.9x10^-4	2.4x10^5	1.2x10^4	1.2x10^2	4.9x10^-2	1.8x10^5	2.7x10^4	5.1x10^3	2.7x10^-2	4.9x10^5	2.7x10^5	1.2x10^5	2.4x10^-1
7	1.6x10^4	1x10^5	0	-	5.7x10^5	1.5x10^4	6.9x10^5	0.1x10^1	9.6x10^5	1.7x10^5	1.8x10^2	1.8x10^-2	1.1x10^5	1.8x10^5	9x10^1	7.6x10^-4	9.6x10^4	8.7x10^4	3.9x10^3	4x10^-2	3.9x10^4	2.7x10^4	4.8x10^3	1.2x10^-1
8	1.2x10^4	2.8x10^7	0	-	5.1x10^5	6.6x10^3	2.4x10^2	4.7x10^-2	5.4x10^4	1.1x10^3	9x10^1	1.6x10^-3	1.2x10^5	1.6x10^4	0	-	1.6x10^5	2.7x10^4	2.4x10^3	1.4x10^-2	4.5x10^4	9.3x10^4	3.6x10^3	8x10^-2
9	6.6x10^5	5.1x10^4	0	-	6.3x10^3	1.7x10^4	5.7x10^2	9.0x10^0	3.3x10^4	2.8x10^4	2.4x10^3	7.2x10^-2	9.9x10^2	3x10^3	0	-	6.3x10^4	3.2x10^4	9x10^3	1.4x10^-1	5.7x10^4	2.1x10^4	6.9x10^3	1.2x10^-1
10	5.1x10^4	6.9x10^4	0	-	3.3x10^3	7.8x10^3	7.5x10^2	2.2x10^-1	2.3x10^4	7.2x10^3	3x10^1	1.2x10^-3	4.8x10^3	5.1x10^4	0	-	1.2x10^5	1.2x10^4	9.9x10^2	7.6x10^-3	7.2x10^4	6.6x10^4	6.6x10^3	9.1x10^-2
11	2.1x10^5	9.9x10^4	0	-	2.7x10^4	3.6x10^4	3x10^1	1.1x10^-3	3.9x10^3	2.1x10^3	3x10^1	7.6x10^-3	2.5x10^3	2.7x10^3	2.4x10^2	9.4x10^-2	2.7x10^3	4.8x10^3	1.2x10^2	4.4x10^-2	1.1x10^5	3.6x10^4	1.2x10^4	1.1x10^-1
12	5.4x10^5	4.8x10^5	0	-	1.3x10^4	2.4x10^3	3.6x10^2	2.6x10^-2	9.6x10^3	4.2x10^5	0	-	3x10^5	5.1x10^3	2.5x10^3	8.4x10^-1	2.7x10^3	7.8x10^3	3x10^1	1.1x10^-2	3.2x10^6	1.9x10^6	1.3x10^5	4x10^-2
13	3.9x10^5	1.4x10^5	0	-	1.2x10^4	2.7x10^4	0	-	6.6x10^4	1.7x10^4	0	-	3.3x10^3	2.7x10^3	6.6x10^2	2x10^-1	2.8x10^3	1.8x10^4	6x10^1	2.1x10^-2				
14	1.2x10^6	9.3x10^5	0	-	1.2x10^5	7.5x10^4	0	-	2.1x10^4	1.2x10^4	0	-	1.3x10^3	1.3x10^3	2.7x10^2	2x10^-1	9x10^3	4.2x10^3	0	-				
15	1.1x10^5	1.4x10^6	0	-	8.1x10^3	1.2x10^4	0	-	1x10^4	3.3x10^3	0	-	5.1x10^3	4.2x10^3	2.7x10^2	5.2x10^-2	6.9x10^3	1.7x10^3	0	-				

Table 3. Horizontal transfer of the antibiotic resistance gene in the house fly labellum (donor first, recipient second)

Fly #	Day 0				Day 1				Day2				Day 3				Day 4			
	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D
1	$1.7x10^{3}$	$2.6x10^{3}$	0	-	$9.6x10^{2}$	$2.9x10^{3}$	0	-	$4.5x10^{2}$	$1x10^{3}$	0	0	0	$1.8x10^{2}$	0	-	$1.1x10^{4}$	$4.5x10^{3}$	$3x10^{1}$	$2.5x10^{-3}$
2	$1.8x10^{3}$	$2.3x10^{3}$	0	-	$3x10^{2}$	$1.4x10^{3}$	0	-	$2.7x10^{2}$	$1.5x10^{3}$	0	0	$4.8x10^{2}$	$3x10^{2}$	0	-	$5.4x10^{2}$	$1.1x10^{3}$	0	-
3	$1.1x10^{3}$	$1.8x10^{3}$	0	-	$1.9x10^{3}$	$4.4x10^{3}$	0	-	$8.1x10^{2}$	$1.5x10^{3}$	0	0	$8.4x10^{2}$	$7.5x10^{2}$	0	-	0	0	0	-
4	$5.4x10^{2}$	$2.7x10^{3}$	0	-	$1.5x10^{3}$	$3.6x10^{3}$	0	-	$6.0x10^{2}$	$2.6x10^{3}$	0	0	$3x10^{2}$	$3.6x10^{2}$	0	-	0	0	0	-
5	$5.4x10^{2}$	$2.1x10^{2}$	0	-	$2.2x10^{3}$	$7.8x10^{4}$	0	-	$7.8x10^{2}$	$1.6x10^{3}$	0	0	$1.5x10^{2}$	$4.8x10^{2}$	0	-	0	0	0	-
6	$3.6x10^{2}$	$1x10^{3}$	0	-	$1.8x10^{2}$	$2.4x10^{2}$	$6x10^{1}$	$3.3x10^{-1}$	$1.5x10^{2}$	$3x10^{3}$	$2.9x10^{3}$	$1.9x10^{1}$	$6x10^{1}$	$4.2x10^{2}$	$5.4x10^{2}$	$0.9x10^{1}$	$7.2x10^{3}$	$1x10^{4}$	$8.1x10^{3}$	$0.1x10^{1}$
7	$8.4x10^{2}$	$1.9x10^{3}$	0	-	$9x10^{1}$	$2.4x10^{2}$	$9x10^{1}$	$0.1x10^{1}$	0	$6.6x10^{2}$	$4.5x10^{2}$	0	$3x10^{1}$	$1.1x10^{3}$	$4.2x10^{2}$	$1.4x10^{1}$	$5.4x10^{2}$	$3.3x10^{3}$	$2.5x10^{3}$	$0.4x10^{1}$
8	$2.4x10^{2}$	$1.3x10^{3}$	0	-	$3x10^{1}$	$1.8x10^{2}$	$9x10^{1}$	$0.3x10^{1}$	0	$7.2x10^{2}$	$4.5x10^{2}$	0	$3x10^{1}$	$1.2x10^{3}$	$1.3x10^{3}$	$4.5x10^{1}$	$9x10^{1}$	$2.4x10^{3}$	$1.8x10^{3}$	$2x10^{1}$
9	$3.6x10^{2}$	$2.8x10^{3}$	0	-	$6x10^{1}$	$1.2x10^{2}$	$2.1x10^{2}$	$0.3x10^{1}$	0	$1.8x10^{3}$	$1.9x10^{3}$	0	$6x10^{1}$	$2.2x10^{3}$	$1.5x10^{3}$	$2.5x10^{1}$	$2.7x10^{2}$	$7.2x10^{3}$	$3.9x10^{3}$	$1.4x10^{1}$
10	$2.1x10^{2}$	$1.8x10^{3}$	0	-	$2.1x10^{2}$	$2.4x10^{2}$	$1.2x10^{2}$	$5.7x10^{-1}$	$1.5x10^{3}$	$1.1x10^{3}$	$8.7x10^{2}$	$5.5x10^{-1}$	$3x10^{1}$	$1.8x10^{3}$	$1.8x10^{3}$	$6.1x10^{1}$	$3.3x10^{2}$	$7.8x10^{3}$	$9.6x10^{3}$	$2.9x10^{1}$
11	$3x10^{1}$	$1.1x10^{3}$	0	-	0	$9x10^{1}$	0	-	$2.1x10^{2}$	$6x10^{1}$	0	-	0	$1.1x10^{3}$	0	-	$6x10^{1}$	$6x10^{1}$	0	-
12	0	$3.9x10^{2}$	0	-	$9x10^{1}$	$1.8x10^{2}$	0	-	0	$3x10^{1}$	0	-	$3x10^{1}$	$2.7x10^{2}$	0	-	$6x10^{1}$	$3x10^{1}$	0	-
13	$6x10^{1}$	$3.3x10^{2}$	0	-	0	$3x10^{1}$	0	-	$3x10^{1}$	$9x10^{1}$	0	-	$2.4x10^{2}$	$2.8x10^{3}$	0	-	0	0	0	-
14	$1.5x10^{2}$	$1.8x10^{2}$	0	-	0	$6x10^{1}$	0	-	$3x10^{1}$	$1x10^{1}$	0	-	$6x10^{1}$	$4.6x10^{3}$	0	-	0	$1.2x10^{2}$	0	-
15	$3x10^{1}$	$1.1x10^{3}$	0	-	0	0	0	-	$3x10^{1}$	0	0	-	$3x10^{1}$	$2.4x10^{3}$	0	-	0	$2.1x10^{2}$	0	-

Table 4. Horizontal transfer of the antibiotic resistance gene in the gut of the surface-sterilized house flies (donor first, recipient second)

Fly #	Day 0				Day 1				Day2				Day 3				Day 4			
	D	R	T	T / D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D
1	$1x10^{5}$	$5.0x10^{4}$	0	-	$1.1x10^{4}$	$2.8x10^{4}$	$3x10^{1}$	$2.6x10^{-3}$	$4.5x10^{3}$	$2.8x10^{4}$	0	0	$5.1x10^{3}$	$1x10^{4}$	$1.1x10^{3}$	$2.2x10^{-1}$	$1.1x10^{5}$	$5.6x10^{4}$	$3x10^{3}$	$2.7x10^{-2}$
2	$4.1x10^{4}$	$3.7x10^{4}$	0	-	$1.1x10^{4}$	$5.6x10^{4}$	$2.4x10^{2}$	$2x10^{-2}$	$3x10^{3}$	$1.5x10^{4}$	$3x10^{1}$	$1x10^{-2}$	$6.3x10^{3}$	$6.4x10^{4}$	$9x10^{1}$	$1.4x10^{-2}$	$5.1x10^{3}$	$2.2x10^{3}$	0	-
3	$3.6x10^{4}$	$3.7x10^{4}$	0	-	$1.1x10^{5}$	$1.3x10^{5}$	$1.9x10^{3}$	$1.7x10^{-2}$	$3.6x10^{3}$	$6.9x10^{3}$	$3x10^{1}$	$8.1x10^{-3}$	$5.7x10^{3}$	$2.9x10^{4}$	0	-	0	0	0	-
4	$5.4x10^{4}$	$5.4x10^{4}$	0	-	$1.9x10^{4}$	$1.2x10^{4}$	0	0	$7.8x10^{2}$	$3.9x10^{3}$	$1.2x10^{2}$	$1.5x10^{-1}$	$3.4x10^{5}$	$1.2x10^{4}$	$3x10^{1}$	$8.6x10^{-5}$	0	0	0	-
5	$1.3x10^{4}$	$1.4x10^{4}$	0	-	$1.5x10^{4}$	$1.6x10^{4}$	$9x10^{1}$	$5.6x10^{-3}$	$7.5x10^{3}$	$1.2x10^{4}$	0	0	$2.7x10^{3}$	$5.1x10^{3}$	$3x10^{1}$	$1x10^{-2}$	0	0	0	-
6	$2.4x10^{3}$	$8.4x10^{4}$	0	-	$1x10^{3}$	$1.2x10^{3}$	$2.7x10^{3}$	$0.2x10^{1}$	$5.7x10^{2}$	$1.2x10^{4}$	$1.7x10^{4}$	$3.1x10^{1}$	$3.3x10^{2}$	$4.6x10^{3}$	$3.3x10^{3}$	$1x10^{1}$	$1.3x10^{4}$	$8.7x10^{4}$	$7.5x10^{4}$	$0.5x10^{1}$
7	$8.4x10^{2}$	$4.8x10^{4}$	0	-	$6.9x10^{2}$	$1.1x10^{3}$	$3x10^{3}$	$0.4x10^{1}$	$3.3x10^{2}$	$1.2x10^{4}$	$8.4x10^{3}$	$2.5x10^{1}$	$6.9x10^{2}$	$1.1x10^{4}$	$5.7x10^{3}$	$0.8x10^{1}$	$3x10^{3}$	$2.1x10^{5}$	$2.1x10^{4}$	$0.7x10^{1}$
8	$1.3x10^{4}$	$2.3x10^{5}$	0	-	$9.9x10^{2}$	$7.2x10^{2}$	$1.8x10^{3}$	$0.1x10^{1}$	$1.9x10^{3}$	$2.2x10^{4}$	$2.2x10^{4}$	$1.1x10^{1}$	$7.2x10^{2}$	$1.4x10^{4}$	$9.9x10^{3}$	$1.3x10^{1}$	$6.9x10^{3}$	$1.4x10^{5}$	$1.4x10^{5}$	$2x10^{1}$
9	$2.3x10^{3}$	$3.9x10^{4}$	0	-	$1.2x10^{3}$	$1.2x10^{3}$	$1.9x10^{3}$	$0.1x10^{1}$	$2.1x10^{2}$	$1.6x10^{4}$	$1x10^{4}$	$5.1x10^{1}$	$8.1x10^{2}$	$2.4x10^{3}$	$1.3x10^{3}$	$0.1x10^{1}$	$7.5x10^{3}$	$1.1x10^{5}$	$9.9x10^{4}$	$1.3x10^{1}$
10	$1.3x10^{4}$	$8.7x10^{4}$	0	-	$1.6x10^{3}$	$2x10^{3}$	$4.8x10^{2}$	$2.9x10^{-1}$	$2.1x10^{3}$	$2.7x10^{4}$	$3.9x10^{4}$	$1.8x10^{1}$	$1.3x10^{3}$	$2.2x10^{5}$	$1.8x10^{5}$	$1.4x10^{2}$	$1.8x10^{3}$	$4x10^{4}$	$5.4x10^{4}$	$2.9x10^{1}$
11	$6.9x10^{2}$	$2.4x10^{3}$	0	-	0	0	0	-	$2.7x10^{2}$	$3.9x10^{2}$	0	-	$3.6x10^{2}$	$1.6x10^{3}$	0	-	$3.3x10^{2}$	$6.4x10^{3}$	0	-
12	$9x10^{1}$	$7.8x10^{2}$	0	-	0	$1.8x10^{2}$	0	-	$2.4x10^{2}$	$6.3x10^{2}$	0	-	$6.9x10^{2}$	$6.6x10^{3}$	$3x10^{1}$	$4.3x10^{-2}$	$3.6x10^{2}$	$7.2x10^{2}$	$6x10^{1}$	$1.6x10^{-1}$
13	$6x10^{1}$	$3x10^{2}$	0	-	$6x10^{1}$	$1.2x10^{2}$	0	-	$3.9x10^{3}$	$2.1x10^{2}$	0	-	$3.6x10^{2}$	$4.8x10^{3}$	$3x10^{1}$	$8.3x10^{-2}$	$1.5x10^{3}$	$9.3x10^{2}$	0	-
14	$1.8x10^{3}$	$1.5x10^{3}$	0	-	0	$6x10^{1}$	0	-	$6x10^{1}$	$3.9x10^{2}$	0	-	$9.3x10^{2}$	$6.6x10^{3}$	0	-	$3.6x10^{2}$	$2.7x10^{3}$	0	-
15	$1.8x10^{2}$	$4.5x10^{3}$	0	-	$3.9x10^{2}$	$1.5x10^{2}$	0	-	$7.5x10^{3}$	$5.1x10^{3}$	$4.8x10^{2}$	$6.4x10^{-2}$	$4.5x10^{2}$	$1x10^{4}$	0	-	$5.4x10^{2}$	$5.7x10^{2}$	0	-

Table 5. Horizontal transfer of the antibiotic resistance gene in the house fly labellum (recipient first, donor second)

Fly #	Day 0				Day 1				Day2				Day 3				Day 4			
	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D
1	$2.6x10^3$	0	0	-	$6x10^1$	$3x10^1$	0	-	$2.1x10^2$	$3x10^1$	0	-	$2.1x10^2$	$6x10^1$	0	-	$2.7x10^2$	$6x10^1$	0	-
2	$2.8x10^3$	0	0	-	$6x10^1$	$6x10^1$	0	-	$6x10^1$	$6x10^1$	0	-	$6.9x10^2$	$2.1x10^2$	0	-	$2.4x10^2$	$6x10^1$	0	-
3	$2.6x10^3$	0	0	-	$2.7x10^2$	$3x10^1$	0	-	0	0	0	-	$3.3x10^2$	$1.5x10^2$	0	-	$3.6x10^2$	0	0	-
4	$2x10^3$	0	0	-	0	0	0	-	0	0	0	-	$7.8x10^2$	$3x10^1$	0	-	0	0	0	-
5	$1.7x10^3$	$1.8x10^2$	0	-	$3x10^1$	0	0	-	0	0	0	-	$2.4x10^2$	$9x10^1$	0	-	0	0	0	-
6	$4.2x10^3$	$2.5x10^3$	0	-	$4.8x10^2$	$7.2x10^4$	0	-	$1.2x10^2$	$7.2x10^2$	0	-	$4.2x10^2$	$4.8x10^2$	0	-	$6x10^1$	0	0	-
7	$4.8x10^3$	$3.7x10^3$	0	-	$2.2x10^3$	$2.4x10^4$	$1.2x10^3$	$5.4x10^{-3}$	$3.6x10^2$	$6x10^1$	0	-	0	0	0	-	0	0	0	-
8	$4.2x10^3$	$2.8x10^4$	0	-	$1x10^3$	$7.2x10^4$	$1.2x10^2$	$1.1x10^{-1}$	$1.2x10^2$	$1.2x10^2$	0	-	0	0	0	-	0	0	0	-
9	$7.8x10^3$	$8.4x10^3$	0	-	$4.2x10^2$	$6x10^3$	0	-	$6.6x10^2$	$9x10^2$	0	-	0	0	0	-	0	0	0	-
10	$6.6x10^3$	$9x10^3$	0	-	$1.3x10^3$	$1x10^5$	0	-	0	0	0	-	0	0	0	-	0	0	0	-
11	$6.9x10^2$	0	0	-	$6x10^1$	$3x10^1$	0	-	$2.1x10^2$	$1.5x10^2$	0	-	$5.1x10^2$	$3x10^1$	0	-	$5.1x10^2$	$3x10^1$	0	-
12	$1.1x10^3$	$1.5x10^2$	0	-	$6x10^1$	$6x10^1$	0	-	$3x10^1$	$3x10^1$	0	-	$2.4x10^2$	$3x10^1$	0	-	$7.2x10^3$	$3x10^1$	0	-
13	$6.9x10^2$	0	0	-	$2.7x10^2$	$3x10^1$	0	-	$2.1x10^2$	$3x10^1$	0	-	$3.3x10^2$	$3x10^1$	0	-	$4.5x10^2$	$6x10^1$	0	-
14	$1.2x10^3$	$3x10^1$	0	-	0	0	0	-	$3x10^1$	$1.2x10^2$	0	-	$2.1x10^2$	$3x10^1$	0	-	$2.1x10^2$	$3x10^1$	0	-
15	$9x10^2$	$3x10^1$	0	-	$3x10^1$	0	0	-	$6x10^1$	$2.1x10^2$	0	-	$2.1x10^2$	$3x10^1$	0	-	$1.8x10^2$	0	0	-

Table 6. Horizontal transfer of the antibiotic resistance gene in the gut of surface-sterilized house flies (recipient first, donor second)

Fly #	Day 0				Day 1				Day2				Day 3				Day 4			
	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D	D	R	T	T/D
1	$1.1x10^5$	$3x10^3$	0	-	$3x10^4$	$8.1x10^3$	0	-	$3.3x10^2$	$3.6x10^2$	0	-	$6.9x10^3$	$9.3x10^2$	0	-	$9.9x10^3$	$1.4x10^3$	0	-
2	$1.1x10^5$	$5.4x10^3$	0	-	$2.3x10^5$	$3.2x10^5$	$1.7x10^3$	$7.5x10^{-3}$	$9.3x10^2$	$8.7x10^2$	0	-	$3.3x10^3$	$6.9x10^3$	0	-	$4.8x10^3$	$8.4x10^2$	0	-
3	$8.6x10^4$	$7.5x10^3$	0	-	$2.7x10^4$	$9x10^2$	0	0	0	0	0	-	$1.5x10^3$	$1x10^4$	0	-	$1.1x10^4$	$9.6x10^2$	0	-
4	$1.4x10^5$	$6x10^2$	0	-	$1.2x10^4$	$1.8x10^3$	0	0	0	0	0	-	$6.3x10^3$	$2x10^4$	0	-	0	0	0	-
5	$1.8x10^5$	$1.9x10^4$	0	-	$6.6x10^3$	$2.7x10^3$	0	0	0	0	0	-	$1.6x10^4$	$8.1x10^2$	0	-	0	0	0	-
6	$1.3x10^5$	$2.7x10^4$	0	-	$6.3x10^3$	$3.6x10^4$	$6x10^1$	$9.5x10^{-3}$	0	0	0	-	$1.0x10^3$	$3.6x10^3$	$6x10^1$	$5.7x10^{-2}$	$1.6x10^3$	$5.5x10^3$	0	-
7	$9x10^3$	$8.7x10^4$	0	-	$3.9x10^3$	$1.2x10^4$	$6x10^1$	$1.5x10^{-2}$	$2.7x10^3$	$2.2x10^4$	0	-	$1x10^5$	$3x10^4$	$9x10^1$	$8.8x10^{-2}$	$4.5x10^2$	$1.7x10^3$	0	-
8	$2.6x10^5$	$1.6x10^5$	0	-	$6.6x10^3$	$3.6x10^4$	0	-	$2.1x10^3$	$6.6x10^3$	0	-	0	0	0	-	0	0	0	-
9	$1.2x10^5$	$4.5x10^4$	0	-	$2.3x10^4$	$3x10^3$	0	-	$3.9x10^2$	$2.7x10^3$	0	-	0	0	0	-	0	0	0	-
10	$1.5x10^5$	$1.3x10^5$	0	-	$1x10^4$	$5.4x10^4$	0	-	$3x10^3$	$9.6x10^3$	0	-	0	0	0	-	0	0	0	-
11	$7.2x10^3$	$1.5x10^4$	0	-	$3.6x10^2$	$2.7x10^2$	0	-	$6x10^2$	$5.7x10^2$	0	-	$2.2x10^3$	$2.7x10^2$	0	-	$7.2x10^3$	$2.5x10^3$	$1.6x10^3$	$2.2x10^{-1}$
12	$3.9x10^4$	$1.8x10^2$	0	-	$3.6x10^2$	$1.8x10^2$	0	-	$9.3x10^2$	$6.9x10^2$	0	-	$7.5x10^4$	$1.2x10^2$	$3x10^1$	$4x10^{-4}$	$1.4x10^4$	$2.2x10^3$	$3x10^1$	$2x10^{-3}$
13	$6x10^2$	$6x10^1$	0	-	$2.1x10^3$	$4.2x10^2$	0	-	$1.3x10^3$	$1.1x10^3$	$2.1x10^2$	$1.5x10^{-3}$	$3x10^3$	$3x10^2$	0	0	$9x10^3$	$1.7x10^3$	$3x10^1$	$3.3x10^{-3}$
14	$1.5x10^4$	$3.9x10^2$	0	-	$3.6x10^2$	$2.1x10^2$	0	-	$2.9x10^3$	$4.8x10^2$	0	-	$7.2x10^3$	$4.8x10^2$	0	0	$6.5x10^3$	$4.5x10^2$	$3x10^1$	$4.6x10^{-3}$
15	$5.1x10^4$	$1.9x10^3$	0	-	$3x10^1$	$1.5x10^2$	0	-	$2.4x10^3$	$4.8x10^3$	0	-	$3.7x10^3$	$9x10^1$	0	0	$1.2x10^3$	$2.7x10^2$	$6x10^1$	$4.6x10^{-3}$